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14. ABSTRACT Breast cancer is the second leading cause of cancer related death for women in the United States. Interestingly, breast cancer cells often highly express the Fas (CD95/APO-1) receptor, which is well established as an activator of apoptosis (programmed cell death) upon ligand binding. Fas induces apoptosis by recruiting proteins that form the death inducing signaling complex (DISC). Type I cells form large amounts of the DISC and internalize Fas, whereas in Type II cells Fas does not internalize and the DISC is almost undetectable. Additionally the Fas receptor has recently been shown to activate the nonapoptotic NF-κB and MAP kinase pathways upon receptor stimulation in either Type I or Type II cells. We can now demonstrate that in Type I cells the recruitment of DISC largely occurs after the receptor has moved into an endosomal compartment and blocking internalization prevents formation of the DISC. Receptor internalization is not required for NF-κB and Erk1/2 activation. Consequently dimerization of Fas complexes does not induce internalization of Fas nor apoptosis but is sufficient to induce nonapoptotic-signaling pathways and increases motility and invasiveness of tumor cells. Monomeric Fas binding in not sufficient to activate nonapoptotic playways. Furthermore, we can demonstrate SNARK's role as a nonapoptotic kinase and promoter of motility and invasion.

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PREFACE

Per the requirements of Department of Defense Breast Cancer Research Fellowship DAMD17-03-1-0200 I am submitting the final report of research preformed as detailed in the updated SOW. This award was officially transferred to me on October 27, 2004. The original recipient, Bryan Barnhart, completed the requirements for his Ph.D, graduated from The University of Chicago, and was not able to complete the experimental tasks detailed in his award. I applied for a transfer of the award on July 19, 2004 and submitted a revised statement of work and subsequently received approval for the transfer of the award on October 27, 2004. Therefore work relating to this project has been discontinuous. As stated in the previous progress report my financial administrator acting as an agent for The University of Chicago filed an amendment of solicitation/modification of contract for a no cost extension on December 29, 2005. I received an approval for the change in the performance period at no cost for a period of one year, extending the termination date form 28 April 2006 to the new termination date of 28 April 2007. Since the financial support from the DOD last until August of 2006 I am now submitting the final report for this fellowship. As my PhD is not yet completed I will continue to work on the project payed from other sources.

INTRODUCTION

Breast cancer cells often express the Fas (Fas/APO-1) receptor, which is well established as an activator of apoptosis upon ligand binding. Fas induces apoptosis by recruiting FADD and initiator caspases 8 and 10 forming a death inducing signaling complex (DISC)[1]. Type I cells form large amounts of the DISC and internalize Fas whereas in Type II cells Fas does not internalize and the DISC is almost undetectable [2-4]. Receptor internalization has been shown to limit extracellular signal stimulation and receptor recycling [2-6]. However, TNF-R1, a death receptor family member, has been shown to internalize upon binding of it ligand [7], TNFα, and TNF-R1 DISC formation requires internalization of the receptor [8]. Activation of the Fas receptor by upregulating the Fas ligand (FasL) is thought to be a desired response to chemotherapy and known to occur in various chemotherapy treatments. However, we and others have observed that Fas receptor stimulation also leads to activation of the nonapoptotic NF-κB and MAP kinase pathways which have known tumorigenic activities [9, 10]. Interestingly, TNF-R1 stimulation leads to activation of nonapoptotic signaling pathways at the plasma membrane and does not require internalization [11]. Both Type I and II cells activate the nonapoptotic pathways following FasL binding [10]. In cases where the receptor has a mutation in the death domain (DD) in only one allele apoptosis induction is blocked, but the nonapoptotic pathways are fully functional [12]. Fas stimulation of the breast cancer cell line MCF7-FB induces up-regulation of a defined number of mostly anti-apoptotic genes, resulting in increased motility and invasiveness of tumor cells [10]. Furthermore, the majority of these genes are known NF-κB target genes. We identified one of the Fas-regulated genes as the serine/threonine kinase (SNF1/AMP kinase related kinase (SNARK)), a member of the AMP kinase family, which is induced in response to various forms of metabolic stress. The mechanism of activation of the nonapoptotic pathways at the Fas receptor level is a primary tenant of this research proposal as described in task 1 and 2 of the SOW. We can now demonstrate that DISC formation requires internalization of Fas in Type I cells, and blocking internalization prevents formation of the

DISC and thus provides an explanation for why the DISC is undetectable in Type II cells. In contrast activation of nonapoptotic pathways through Fas, such as NF-κB and Erk1/2, do not require receptor internalization. Furthermore, receptor dimerization is sufficient to activate the nonapoptotic pathways and to increase motility and invasiveness of MCF7-FB cells, but incapable of causing internalization and activation of the apoptotic response. Additionally, we show that over expression of SNARK renders tumor cells more resistant, whereas a kinase-inactive mutant of SNARK sensitizes cells to Fas-mediated apoptosis. Furthermore, small interfering RNA-mediated knockdown of SNARK increased the sensitivity of tumor cells to FasL- and TRAIL-induced apoptosis. Importantly, cells with reduced expression of SNARK also showed reduced motility and invasiveness in response to Fas engagement. SNARK therefore represents an NF-κB-regulated anti-apoptotic gene that contributes to the tumor-promoting activity of Fas in apoptosis-resistant tumor cells. The following details work complete and in progress that directly relates to the SOW.

RESEARCH

Task 1. Determine the mechanism of activation of NF-kB and MAP kinases by Fas at the receptor level

My original approach to this problem as stipulated in the SOW was to stimulate the Fas receptor with monoclonal bivalent antibody fragments $F(ab')_2$ and/or the monoclonal monovalent antibody fragment (Fab). I completed the generation of monovalent Fab fragments and the characterization of the activities arising from monomeric Fas binding. Additionally, we were able to take advantage of current commercially available antibodies against Fas that do not form higher aggregates and tested the activity of the stimulated dimmer; furthermore, we employed a newly described technique to isolate stimulated Fas receptor complexes, resulting in interesting data. The information gained was such that we and our collaborators have published this work.

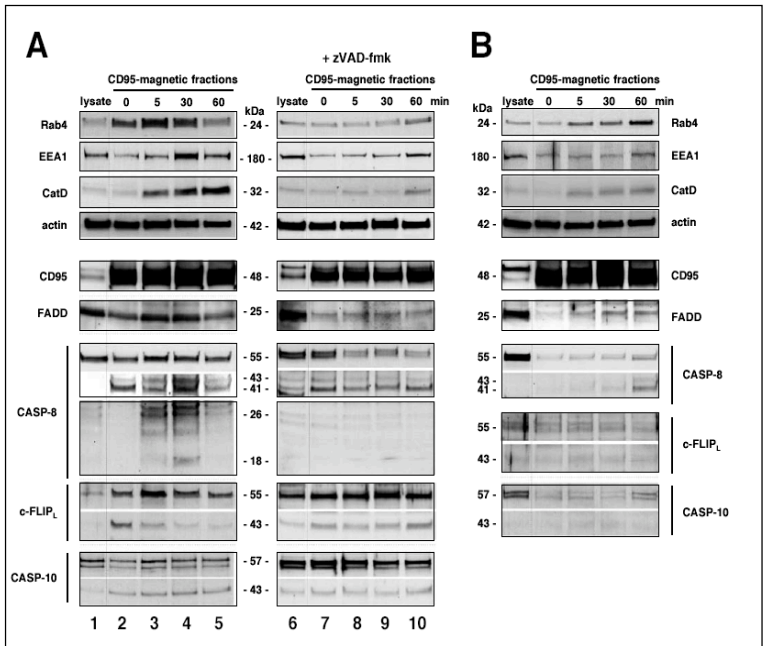

Fig 1. Internalization and endosomal maturation of Fas receptors and recruitment of FADD, caspase-8, cFLIPL and caspase-10 to Fas magnetic fractions isolated from ACHN and HCT15 cells. (**A**) Time course of intracellular Fas-receptosome trafficking in ACHN cells and effect of zVAD-fmk. Total cell lysates or magnetic fractions derived after 0, 5, 30 and 60 min of anti-APO-1 mAb treatment in the absence or presence of zVAD-fmk were analyzed for signature proteins of endosomal maturation (Rab4, EEA1) and lysosomes (32 kDa CatD), actin, Fas, and the DISC composed of FADD, caspase- 8, c-FLIPL and caspase-10 by Western blotting. (**B**) Time course of recruitment of endosomal, lysosomal and the DISC proteins to Fas magnetic fractions isolated from HCT15 cells.

In Type I cells the DISC forms following receptor internalization: We first wanted to determine the order in which the DISC forms with respect to the internalization events.

We took advantage of a novel method used to identify the requirement for internalization of TNF-R1 in the formation of the DISC [8]. We selected cells lines from a group or 22 tumor cells lines recently classified as Type I or Type II - Type I cells form large amounts of the DISC, whereas Type II cells do not [2]. Our choice of solid tumor cells lines HCT15 (Type II) and ACHN (Type I) was based on their high levels of Fas expression. ACHN (Figure 1A) and HCT15 (Figure 1B) cells were incubated with biotinylated anti-APO-1 mAb. The labeled antibody was isolated and with it intact intracellular vesicles to which Fas was localized as previously described [8] at different time points post stimulation. Western blotting for marker proteins typical of endosomal/lysosomal vesicles was used to detect the molecules associated with the isolates. This method allowed us to follow the movement of labeled receptor through the endosomal/lysosomal pathway. Maximum detection of Rab4, the marker for endosomal trafficking, occurred at 5 min in ACHN cells, marking the time point at which the receptor began to internalize (Figure 1A, lanes 1-5). The early endosomal marker protein, endosomal autoantigen 1 (EEA1), appeared at 5 min and peaked at 30 min indicating that the vesicles containing the activated Fas had moved inside the cell and arrived in an endosomal compartment. The lysosomal marker cathepsin D began to appear at 5 min and kept increasing until 60 min indicating that Fas and its associated proteins moved into lysosomes as early as 5 min after stimulation (Figure 1A, lanes 1-5, top panels). In contrast to the Type II cell line HCT15 where all three marker proteins increased only slightly 60 min after stimulation indicating a lack of significant internalization by activated Fas. We then analyzed the isolates for the presence of recruited DISC components. In ACHN cells recruitment of FADD, caspase-8, caspase-10 and activation of caspase-8 peaked at 30 min, a time point at which most of the receptor had moved into an EEA1 containing compartment. Only c-FLIP$_L$ was found to be recruited and cleaved earlier. The data demonstrate that the major recruitment of the DISC components occurs after the onset of internalization of Fas in ACHN cells, and no significant recruitment of DISC components to activated Fas was detected in HCT15 cells during the course of the experiment consistent with the inability of these cells to internalize Fas (Figure 1B).

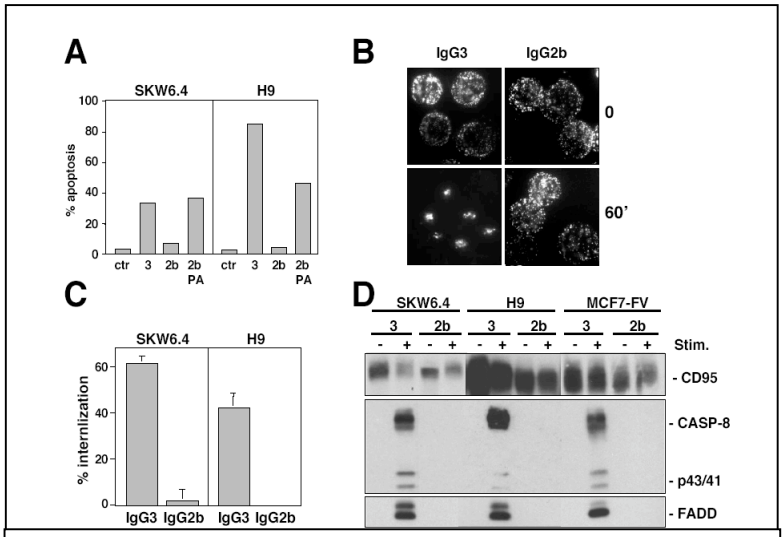

Fig 2. The IgG2b anti-APO-1 switch variant does not induce internalization or death inType I cells. (**A**) DNA fragmentation of cells treated with control IgG (ctr), anti-APO-1 IgG3 (3) or anti-APO-1 IgG2b (2b) without or with 100 ng/ml protein A (PA) added. The experiment was done in triplicates. Shown is the mean with standard deviation. (**B**) Clustering of Fas in SKW6.4 cells after stimulation with FITC-conjugated anti-APO-1 IgG2b or IgG3 for 1 hour. (**C**) Quantification of the number of cells with internalized Fas after 1 hr of stimulation with either IgG3 or IgG2b anti-APO-1. The experiment was done in triplicates. Shown is the mean with standard deviation. (**D**) Standard DISC analysis of SKW6.4, H9 and MCF7-FV cells stimulated with anti-APO-1 IgG3 or IgG2b.

It has been shown that internalization of Fas was block in Type I cells by inhibiting the activity of caspase-8 [13, 14]. Therefore inhibiting the activity of caspase-8 should prevent the formation of the DISC. Repeating the experiment with ACHN cells

in the presence of zVAD-fmk, a caspase inhibitor (Figure 1A, right panel), we observed that internalization of Fas was blocked in these cells and there was no recruitment of the DISC components. The observation is such that zVAD-fmk treated ACHN cells behaved much like the HCT15 cells with only a very late and minor recruitment of DISC components to the activated receptor. Our data suggest that activated Fas requires internalization for the recruitment of most of the apoptotic signaling molecules in Type I cells. When internalization is blocked none of the DISC components are efficiently recruited by Fas, a situation very similar to that found in Type II cells which do not internalize Fas.

IgG2b anti-APO-1 does not induce clustering, internalization, DISC formation, or apoptosis: DISC formation in Type I cells requires the internalization of Fas to fully activate the apoptotic pathway. Type I and Type II cells also activate nonapoptotic pathways, NF-κB and MAPK, upon Fas stimulation. This is true for many types of tumor cells including breast cancer cells [10]. Type II cells do not internalize Fas and it has been previously observed that NF-κB is activated upon Fas stimulation under conditions that inhibit receptor internalization [2]. Furthermore, monoallelic death domain mutations are thought to block apoptotic signaling by preventing trimerization of the aggregate receptors [15]. We sought to determine whether dimeric activation of the receptor was insufficient to cause apoptosis. The anti-APO-1 IgG3 antibody is thought to cause receptor aggregation and subsequent activation of apoptosis via Fc-Fc interactions [16]. The IgG2a or b isotype has the same specificity for Fas but lacks the ability to promote Fc-Fc interactions. Therefore IgG2b binding to Fas will be limited to dimerization without aggregation. Consistent with previous data we found that the IgG2b antibody was insufficient to cause apoptosis of SKW6.4 and H9 cells until the antibody was crosslinked by protein A (Figure 2A). To test whether the nontoxic anti- APO-1 IgG2b isotype could induce clustering and internalization of Fas on Type I cells we added FITC conjugated anti-APO-1 IgG2b to SKW6.4 and H9 cells. This antibody did not induce any detectable clustering of Fas (Figure 2B and data not shown), and no internalization could be detected in cells treated with the IgG2b antibody (Figure 2C). The IgG2b anti-APO-1 Ab did not induce formation of the DISC (Figure 2D), which is consistent with our previous data for the requirement of Fas internalization in the DISC formation.

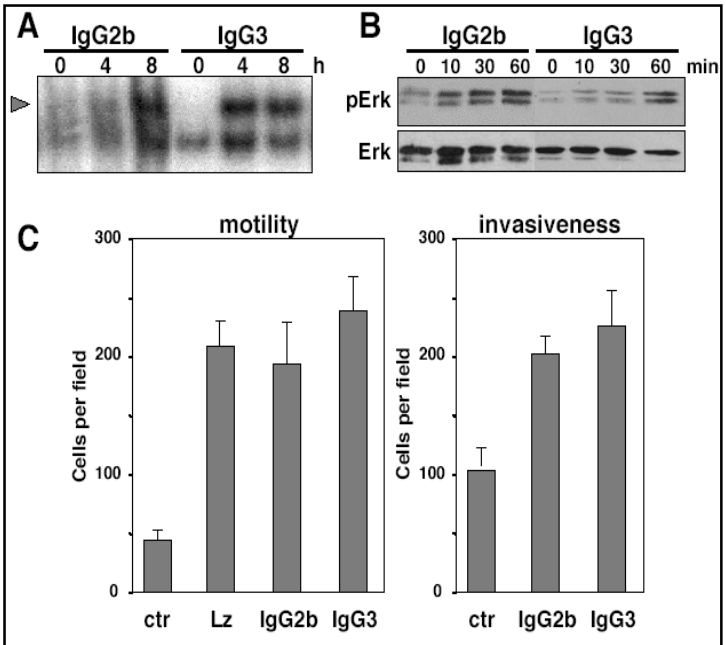

Fig 3. Dimerizationn of Fas complexes is sufficient to induce nonapoptotic pathways through Fas. (**A**) EMSA analysis of MCF7-FV cells stimulated with IgG2b anti-APO-1 and MCF7-FB cells stimulated with IgG3 anti-APO-1 for the indicated times. The arrowhead marks the migration of the p50/p65 NF-κB heterodimer. (**B**) MCF7-FV cells stimulated with IgG2b anti-APO-1 and MCF7-FB cells stimulated with IgG3 anti-APO-1 for the indicated times were subjected to Western blot analysis for phospho-Erk (pErk) and Erk. (**C**) MCF7-FB cells were treated with either control IgG (ctr), LzFasL (Lz), IgG2b or IgG3 anti-APO-1 were subjected to either a motility (left) or Invasiveness (right) assay.

These data suggest that divalent antibody induced dimerization of preassociated Fas complexes is insufficient to trigger clustering or internalization of Fas, formation of the DISC or apoptosis through Fas.

IgG2b anti-APO-1 induces activation of NF-κB and MAPK: IgG2b binding is insufficient cause internalization and apoptosis. We therefore tested whether the antibody could activate Fas stimulated nonapoptotic pathway. MCF7-Fas Vector (MCF7-FV) cells which have been shown to form a DISC when stimulated with IgG3 anti-APO-1 and efficiently activate nonapoptotic pathways when stimulated through Fas, showed no clustering, internalization or formation of the DISC when stimulated with IgG2b anti-APO-1 (data not shown and Figure 2D). However this Ab induced activation of NF-kB and Erk1/2 in MCF7-FV cells as effectively as the IgG3 isotype in apoptosis resistant MCF7-FB cells (Figure 3A and B) suggesting that dimerization of Fas complexes at the cell surface is sufficient to activate nonapoptotic pathways.

IgG2b anti-APO-1 induces motility and invasiveness: Finally we tested whether stimulation of tumor cells with the IgG2b anti-APO-1 Ab would induce an increase in in vitro motility and invasiveness (Figure 3C). Both of these responses were observed when MCF7-FB cells were incubated with the IgG2b Ab. Our data suggest that signaling through Fas causes tumor cells to become more invasive when the signal originates from cell surface and does not require formation of the DISC or the internalizing receptor. Consistent with this conclusion is the observation that we did not find any difference in Fas induced activation of nonapoptotic pathways between Type I and Type II cells (data not shown).

Monovalent anti-APO1 antibody fragment binds to Fas but does not induce apoptosis. The observation that divalent binding of Fas is sufficient to activate the nonapoptotic pathways but does not cause internalization of Fas or activates the apoptotic pathways leaves us with no understanding of what are the minimal binding requirements of Fas to activation the nonapoptotic pathways. It has previously been reported that upon binding monovalent Fab fragments can activate certain intracellular pathways [17] and the monovalent binding resulting from a single epitope recognition in the E rosette receptor (CD2) activates Ca^{2+} release and mitogenic activity [18]. Furthermore, it was found that increasing the valency from the monovalent Fab to the divalent $F(ab')_2$ and sepharose crosslinked $F(ab')_2$ against CD3 increases the receptor activity and results in a change in the signaling pathways and cellular response with the increased binding valency [18].

I sought to address the possibility that monovalent Fas binding may be sufficient to activate nonapoptotic pathways. The best method to answer this question was to incubate Fas expressing cells with a monovalent Fab fragment generated from anti-APO1-IgG3 antibody (MW~155kD). I generated the monovalent Fab fragment (MW~25kD) by utilizing a papain digestion method from the commercially available ImmunoPure Fab Prepartation Kit (cat. 44885) produced by Pierce Biotechnology and illustrated in figure 4. The eluted product was then quantified and 1μg run on 12% SDS-PAGE and transferred to nitrocellulose for western blot analysis to determine if there was any detectable heavy chain present. There was no detectable Fc (heavy chain) region observed from the Fab fragment in either silver stain or using an anti-mouseIgG3(Fc)-HRP antibody, demonstrating that digestion of the Ab was complete(Fig 5a and b). FACS analysis,

using an anti-mIgG-L_κ-FITC antibody determined that Fab binding activity was similar to anti-APO1-IgG3 in MCF7-FB (Fig 5C) with a I sought to address the possibility that monovalent Fas binding may be sufficient to activate nonapoptotic pathways. The best method to answer this question was to incubate Fas expressing cells with a monovalent Fab fragment generated from anti-APO1-IgG3 antibody (MW~155kD). I generated the monovalent Fab fragment (MW~25kD) by utilizing a papain digestion method from the commercially available ImmunoPure Fab Prepartation Kit (cat. 44885) produced by Pierce Biotechnology and illustrated in figure 4. The eluted product was then quantified and 1µg run on 12% SDS-PAGE and transferred to nitrocellulose for western blot analysis to determine if there was any detectable heavy chain present. There was no detectable Fc (heavy chain) region observed from the Fab fragment in either silver stain or using an anti-mouseIgG3(Fc)-HRP antibody, demonstrating that digestion of the Ab was complete(Fig 5a and b). FACS analysis, using an anti-mIgG-L_κ-FITC antibody determined that Fab binding activity was similar to anti-APO1-IgG3 in MCF7-FB (Fig 5C) with a mean fluorescence intensity (MFI) of 64.9±4.4 for the Fab treated cells and 93.4±4.5 for the anti-APO1-IgG3 treated cells compared to an MFI of 5.5±0.4 for the isotype control, indicating efficient binding of the Fab fragment to the Fas receptor. Crosslinking the Fab fragment led to a significant increase in apoptosis as observed by Nicoletti assay when compared to the uncrosslinked monovalent Fab in SKW6.4 cells (fig 5d).

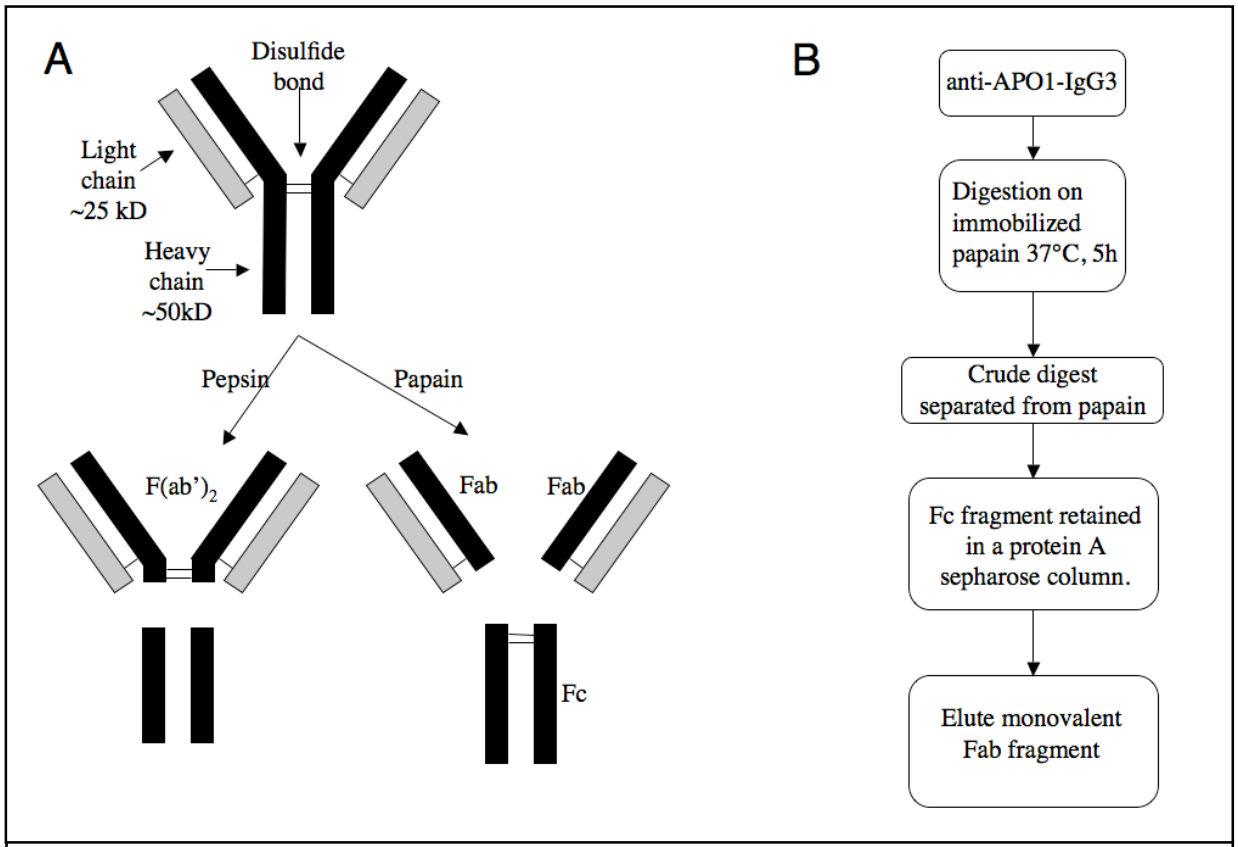

Figure 4 Scheme for the generation of a monovalent Fab fragment. Anti-APO1-IgG3 antibody was subjected to papain digestion and the Fc fragments retained on protein-A sepharose column per the protocol for the ImmunoPure Fab Preparation Kit (cat. 44885, Pierce Biotechnology).

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Monevalent anti-APO1 antibody fragment does not induce activation of apoptotic or non-apoptotic signaling pathways nor does it induce cell motility. I have established the binding of the Fab fragment to Fas but I have yet to describe any functional consequence of the monovalent binding. Therefore I treated MCF7-FB cells with an equimolar amounts of antigen recognition sites, 0.67μg/mL of the Fab fragment or 1μg/mL anti-APO1-IgG3 or 1μg/mL LzFasL and compared the activation of nonapoptotic pathways Erk and NF-κB. Figure 6A illustrates that Erk activity was unchanged after 1h Fab incubation but similar to previously reported activity with LzFasL and anti-APO1-IgG3. Additionally, EMSA analysis indicated no activation of the NF-κB pathway (Fig 6B). Concurrently and as expected there was no increase in motility in MCF7-FB treated cells (Fig 6C). Therefore we can conclude that monomeric binding is insufficient to activate nonapoptotic pathways and that Fas dimerization is the minimal requirement to activate the nonapoptotic pathways activation through Fas.

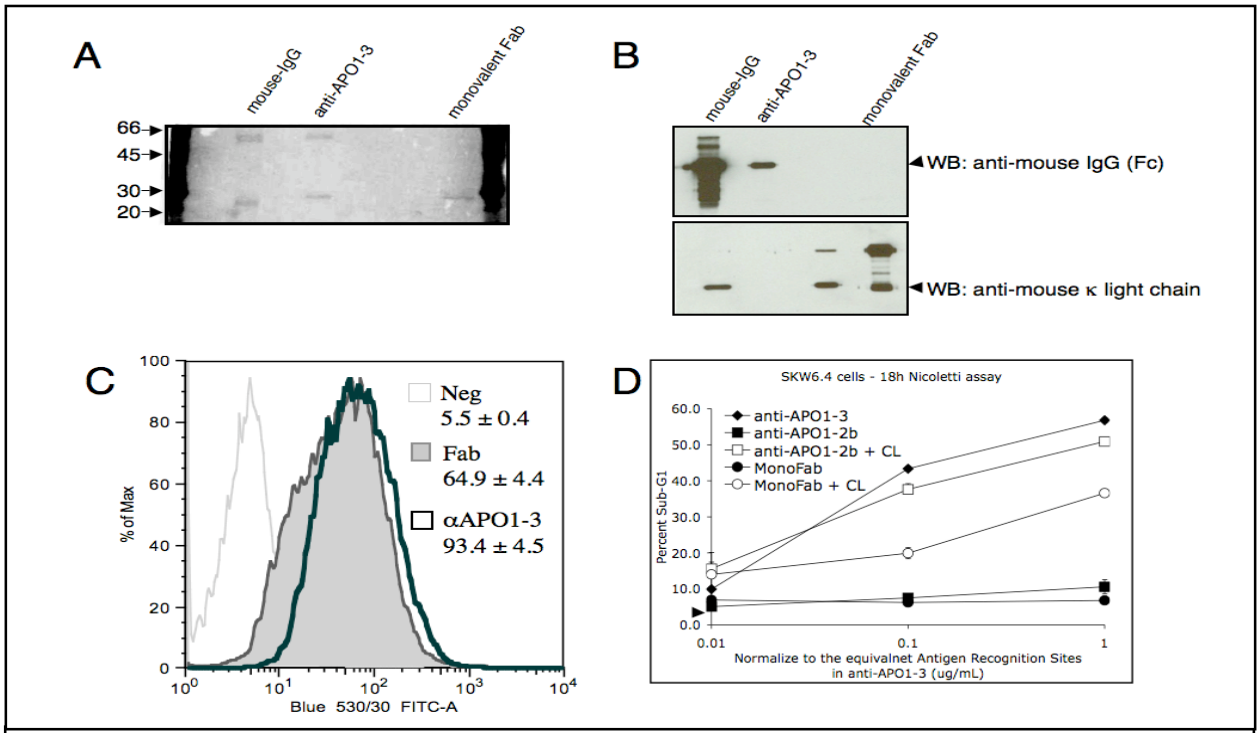

Figure 5 anti-APO1 Fab fragment characterization. A) Silver stain of 12%SDS-PAGE loaded with 1μg of mIgG, α-APO1-IgG3, or eluted Fab fragment. B) Detection of IgG3 heavy chain (Goat anti-Mouse IgG (Fc), Fischer Scientific, cat. pc-31437) and light chain (Rat anti-Mouse Kappa Chain–HRP, Introgen, cat. 04-6620) by western blot analysis of 1μg mIgG, α-APO1-IgG3, or Fab fragment. C) FACS analysis of MCF7-FB cells incubated with 5μg/mL of mIgG3, α-APO1-IgG3, or the monovalent Fab fragment using an α-mIgG-$L_κ$-FITC antibody. D) Nicoletti assay of SKW6.4 cell following 18h incubation with αAPO1-IgG3, αAPO1-IgG2b, or the Fab Fragment ± αmIgG-L chain crosslinking antibody.

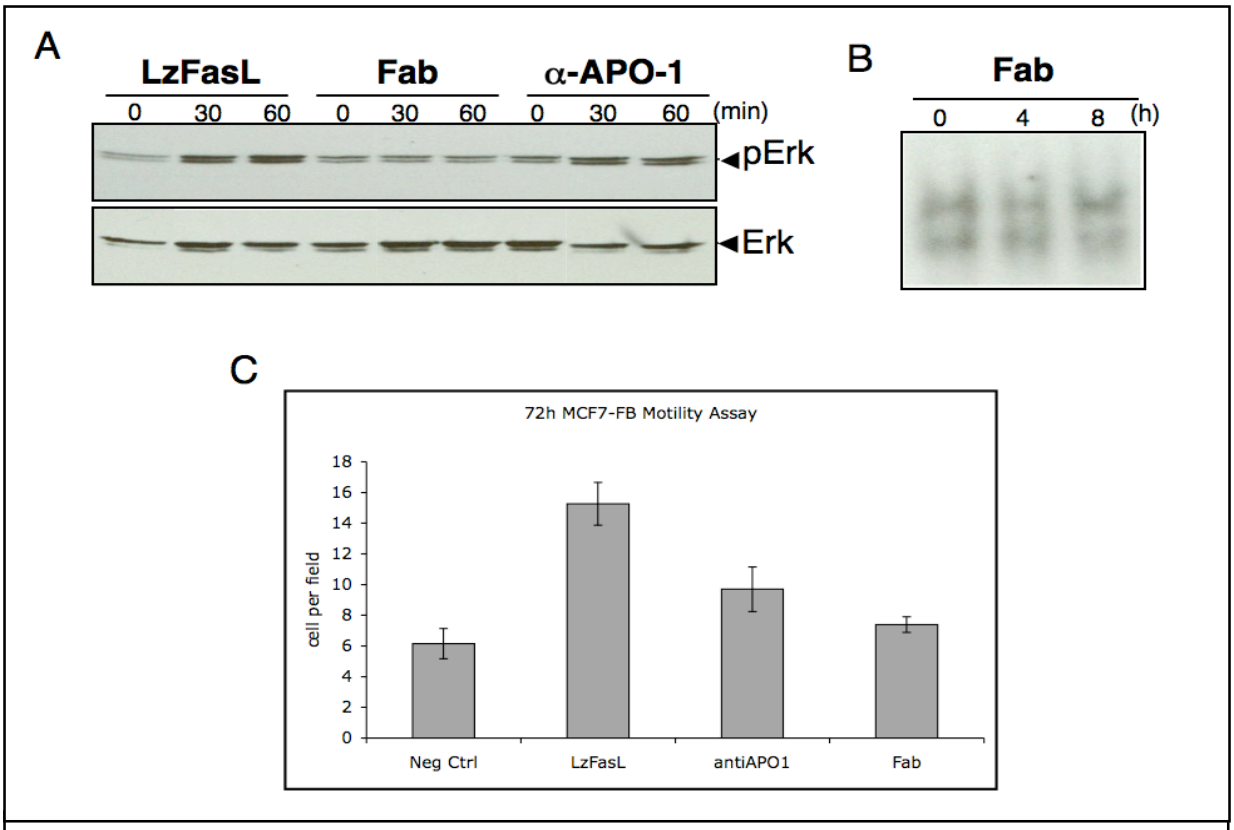

Figure 6 Fab fragment does not activation Fas mediated nonapoptotic pathways. MCF7-FB cells were stimulated as indicated with 1ug/mL LzFasL, 0.67ug/mL Fab fragment, or 1ug/mL anti-APO1-3 and analysed for Erk activation (A), NF-κB activty (B) , or ability to induce motility in a transwell migration assay.

Task 2. Test known Fas signaling molecules as mediators of activation of NF-κB and MAP kinases by Fas .

The issues relating to Task 2 is actively being address as stated in the SOW. I expect preliminary results over the next few months. I previously stated the last update that we were planning a wide scale screen approach to the problem in an attempt to identify the molecular mediators for the activation of the nonapoptotic pathways unique to Fas stimulation. My plan was to employ the use of an siRNA library to screen and identify molecules that selectively upregulate NF-κB in Fas when compared to TNFα stimulation. While all the preliminary work to establish the system has been completed since the last update using an NF-κB driven GFP reporter to observe the activity of NF-κB in transfected MCF7-FB cells (data not shown), we have had to reevaluate our position in the experiment. Our collaborators in this project have changed the direction of their research interests and the resulting experimental costs are quite prohibitive for any single lab. However, this approach is readily viable and the system is functional should the resources or an additional collaborator become available. Until such resources are available I will continue this work and revert to the original approached as desribed in the SOW.

Task 3. Determine the importance for SNARK for Fas induced increase of invasiveness in breast cancer cells.

The majority of task 3 was reported the previous update and there is little change noted in this update. We have published the results pertaining to SNARK. Parts of task 3a and all of task 3b have been completed. I will begin work on the uncompleted issues describe in task 3 for later in the year after the end of the support by the DOD.

Kinase Inactive SNARK Sensitizes Cells to Fas Mediated Apoptosis - We overexpressed the cloned human SNARK kinase in 293T cells and determined that it is an active kinase with typical autophosphorylation properties and with an apparent size of 70 kDa (Fig. 7A). We also detected a phosphorylated protein in control transfected cells that comigrated with immunoprecipitated untagged SNARK likely corresponding to active endogenously expressed SNARK (Fig. 7A, lane 5). To determine the function of endogenous SNARK in Fas induced apoptosis we generated a kinase inactive mutant (Fig. 7B). This mutant was generated by replacing lysine 82, a highly conserved position in the AMP kinase family, with arginine. This residue change has been shown to act in a dominant negative fashion when overexpressed. The kinase function of the K82R mutant of SNARK was inactive as it did not undergo autophosphorylation (Fig. 7B) or phosphorylate a substrate comprising a fusion protein of GST with the SAMS peptide (Fig. 7C), the optimal sequence for phosphorylation by members of the AMP kinase family [19]. Overexpression of either SNARK or SNARK K82R in the Type I cell line ACHN and stimulation of Fas resulted in the cells being significantly protected from apoptosis by the expression of SNARK (Fig 7D). This inhibition was dependent on the kinase activity of SNARK since SNARK K82R could not protect cells from Fas mediated apoptosis. In

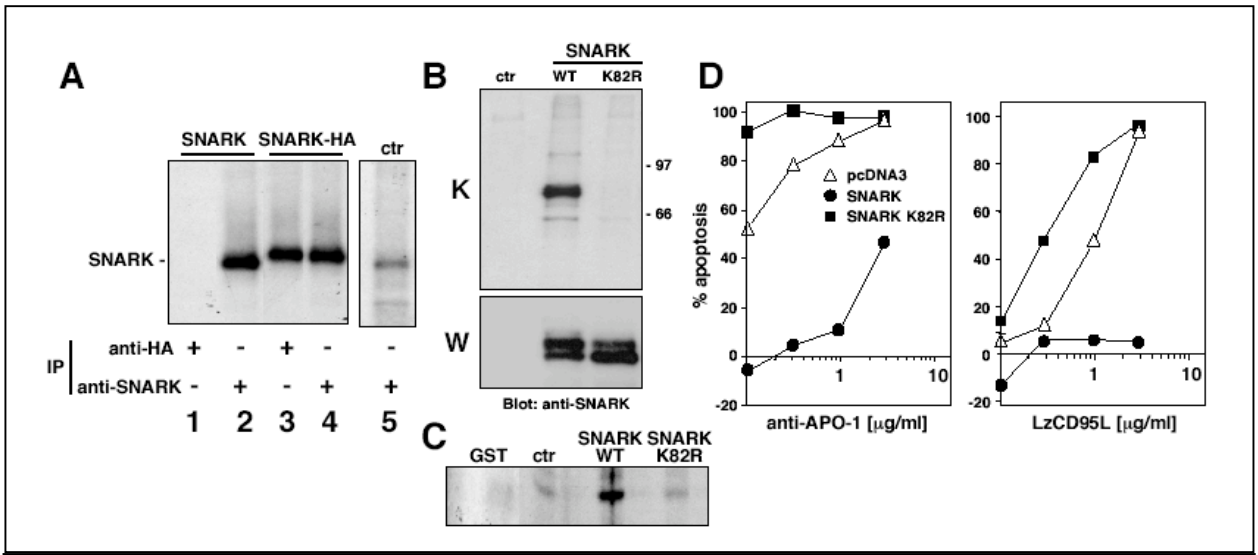

Fig 7. SNARK inhibits Fas induced apoptosis. *A, In vitro* kinase assay of SNARK or HA-SNARK immunoprecipitated with the indicated antibodies from lysates of transiently transfected 293T cells. *B*, 293T cells were transfected with wild-type or K82R mutant SNARK and an autophosphorylation assay (K) was performed. Equal expression of SNARK and the mutant protein in 293T lysates was controlled by an anti-SNARK immuno-blot (W). *C, In vitro* kinase assay on the SAMS peptide. Lysates of empty vector (ctr), SNARK wild type or K82R SNARK transfected 293T cells were used to perform a kinase assay using GST-SAMS as substrate. The same amount of GST alone was incubated with the SNARK wild-type transfected 293T lysate and used as control to demonstrate the specific phosphorylation of SAMS peptide. *D,* HuSNARK inhibits Fas-induced apoptosis and kinase-dead SNARK (SNARK K82R) sensitizes ACHN cells to Fas induced apoptosis. ACHN cells were transfected with the indicated constructs and stimulated 16 hours with the indicated stimuli. The quantification of cell death was performed with a MTS assay in triplicates.

contrast, SNARK K82R rendered cells even more susceptible to Fas mediated apoptosis suggesting that endogenous SNARK was dominant negatively inhibited by the overexpression of mutant SNARK. Transient overexpression of SNARK also protected another Type I cell lines CAKI-1 from both Fas ligand (FasL) and TRAIL induced apoptosis (data not shown).

SNARK Is an Antiapoptotic Gene Required for Fas-induced Increase of Motility and Invasiveness of Fas Mediated Apoptosis Resistant Tumor Cells - To directly determine the function of SNARK as a Fas regulated gene we reduced the expression of SNARK using siRNAs. RT-PCR of ACHN cells found endogenous expression of SNARK (Fig. 8A, lane 1); however expression could be enhanced by treating cells with LzFasL (Fig. 8, lane 2). Treating the cells with two independent siRNAs (siSNARK and an siRNA SMART pool) for 48 hrs resulted in a significant reduction of SNARK mRNA (Fig. 8A, lanes 3 and 4). ACHN cells showed increase sensitivity to Fas (Fig. 7B) or TRAIL (Fig. 8C) mediated apoptosis when treated with the SNARK siRNA. These experiments confirmed the antiapoptotic activity of endogenous SNARK for death receptor induced apoptosis. SNARK was one of only 17 Fas induced genes in MCF7-FB cells, which respond to Fas triggering with increased motility and invasiveness [10]. We therefore tested whether the loss of SNARK would inhibit the Fas mediated migration and invasiveness characteristic of these cells. As reported previously MCF7-FB cells responded to stimulation with LzFasL with upregulation of typical NF-κB target genes such as urokinase plasminogen activator (uPA), A20 and of SNARK (Fig. 8D, lanes 1 and 2). Treating the cells with either of the siRNAs directed at SNARK mRNA resulted in a significantly reduced upregulation of SNARK without affecting induction of uPA or A20 (Fig. 8D, lanes 3 and 4). We then subjected siRNA SNARK-treated MCF7-FB cells to motility and invasiveness assays. Both Fas induced

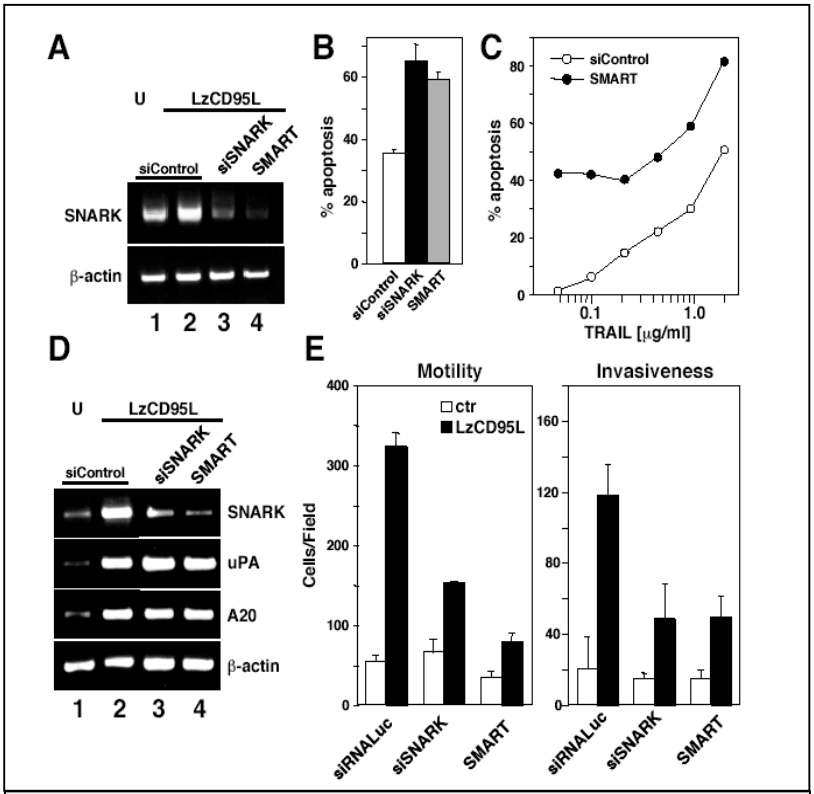

Fig 8. SNARK knock-down increases the sensitivity toward FasL and TRAILtriggered apoptosis and inhibits Fas-induced motility and invasiveness. *A*, Semiquantitative RT-PCR of ACHN cells transfected with a siRNA control (siRNA Luciferase-Cy3) or with two different SNARK specific siRNAs for 24 hours (see experimental procedures), left untreated (U) treated or Treated for two hours with LzFasL. Cells were pre-incubated 1 hour with 40 μM of zVAD-fmk. *B, C*, ACHN cells were transfected with siRNA control or siRNA SNARK and after 24 hours incubated for 16 hours with 1 μg/ml of LzFasL (B) or the indicated concentrations of TRAIL (C) and cell death was quantified by a MTS assay. *D*, Semi-quantitative RT-PCR of MCF7-FB cells transfected with a siRNAs and treated with LzFasL as in A. *E*, The MCF7-FB cells were transfected with the indicated siRNA constructs and after 24 hours were subjected to *in vitro* motility and invasiveness assays.

motility and invasiveness were severely reduced in siSNARK treated cells without affecting the general viability of these cells (data not shown), identifying SNARK as a gene that regulates this novel nonapoptotic activity of Fas.

Both AMPK and another member of the AMPK family, ARK5, which shows 55% overall homology with SNARK, including 84% identity within the N-terminal kinase domain, were recently described as having antiapoptotic activity [20, 21]. Overexpression of ARK5 rendered cells more resistant to TNFα, TRAIL, and glucose deprivation. It was therefore postulated that ARK5 could promote tumor cell survival during nutrient starvation. The recent recognition that Fas has a tumorigenic activity on tumor cells both in vitro [10] as well as in vivo [22] suggests the existence of protumorigenic genes that are induced in response to Fas stimulation. SNARK induction requires activation of NF-κB, which was shown to be critical for the Fas protumorigenic activities.

Task 3 Outlook: I am working on the generation of stably transfected SNARK and dominant negative (dn) SNARK in various cell lines. Once we have established this I will repeat the transfections in murine cells. I have already completed RT-PCR of murine lung cancer cell line 3LL and confirmed the presence of the murine ortholog of SNARK. Preceding the in-vivo observations of the effect of SNARK and dn-SNARK in mice, I will characterize the transfected cells lines to see if the previous observations in human cells are recapitulated in the murine system.

CONCLUSIONS

The research focus of this project deviated from the time frame delineated in the updated SOW do to the transfer of the grant and receipt of a no-cost extension. The minor deviations from the original outline provided us with valuable information pertaining to the mechanisms of Fas mediated signaling. In summary, Type I cells require internalization of Fas for formation of the DISC and efficient induction of apoptosis. In contrast to Type II cells, which do not require internalization to induce apoptosis. Activation of nonapoptotic signaling pathways by Fas generally does not require receptor internalization regardless of whether cells are Type I or Type II and dimerization of receptor complexes is sufficient to induce activation of NF-κB and MAP kinases whereas monomeric binding is insufficient to activate these pathways. These data suggest that signaling pathways emanating from Fas are initiated by signaling complexes that differ not only quantitatively but also in their subcellular localization. Nonapoptotic signaling pathways can be activated through nonaggregated receptors at the plasma membrane but only upon divalent binding and not monovalent. This is in contrast to the apoptotic signaling observed in Type I cells which requires aggregation of Fas and movement of the activated receptor into plasma membrane derived endosomes to recruit sufficient amounts of FADD and caspase-8 needed for cell death. This novel pathway to activate caspase-8 may be important for targeting the caspase to its intracellular substrates. Additionally, our lab recently demonstrated that uPA, one of the NF-κB regulated genes induced by Fas, is critical for the Fas induced motility and invasiveness of apoptosis resistant breast cancer cells cells. Blocking the activity of uPA blocked the ability of cells to invade in response to Fas stimulation. SNARK is the second of the recently

identified Fas induced genes tested and siRNA induced down-modulation of SNARK demonstrated SNARK's importance for the novel Fas dependent tumorigenic activities. Reducing the expression of SNARK did not affect the expression of uPA suggesting that SNARK is not required for the induction of uPA. The fact that Fas activates at least 5 different nonapoptotic signaling pathways independently resulting in induction of multiple genes suggests that the regulation of these activities is complex. The outlook for future research in Fas mediated tumorigenisis has enormous potential and has evolved into my doctoral thesis proposal. This unique and diverse signaling process has many questions that demand to be addressed. The answers yet to be found may prove to be extremely beneficial not only to women suffering from breast cancer but to all people who suffer from cancer.

KEY RESEARCH CONCLUSIONS

- DISC formation in Fas stimulated Type I cells requires internalization
- Fas receptor dimerization is insufficient for DISC formation
- Fas receptor dimerization is sufficient for activation on nonapoptotic pathways
- Fas receptor dimerization is sufficient to induce motility and invasion
- Fas receptor monomeric binding is insufficient to induce activation of nonapoptotic pathways.
- SNARK is acts as antiapoptotic kinase in Fas stimulated cells
- SNARK is required for Fas induced motility and invasion

REPORTABLE OUTCOMES

Publications:

Lee, K.H., Feig, C., Tchikov, V., Schickel, R., Hallas, C., Schuetze, S and Peter, M.E., Chan A.C., The role of receptor internalization in CD95 signaling. **EMBO**, 2006 Mar 8;25(5):1009-23

Park, S.M., Schickel, R., Peter, M.E., Nonapoptotic functions of FADD-binding death receptors and their signaling molecules. **Curr Opin Cell Biol.** 2005 Dec;17(6):610-6

Legembre, P., Schickel, R. Barnhart, B.C. and Peter, M.E. (2004) Identification of SNF1/AMPK-related kinase as a NF-□B regulated antiapoptotic kinase involved in CD95-induced motility and invasiveness. **J Biol Chem**, 279, 46742-46747.

Presentations:

Era of Hope – Department of Defense Breast Cancer Research Program June 2005 (Poster)
The University of Chicago Committee on Cancer Biology, Works in Progress, April 2006
The University of Chicago Committee on Cancer Biology, Works in Progress, April 2005

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Identification of SNF1/AMP Kinase-related Kinase as an NF-κB-regulated Anti-apoptotic Kinase Involved in CD95-induced Motility and Invasiveness*

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The death receptor CD95 (APO-1/Fas) induces apoptosis in many tissues. However, in apoptosis-resistant tumor cells, stimulation of CD95 induces up-regulation of a defined number of mostly anti-apoptotic genes, resulting in increased motility and invasiveness of tumor cells. The majority of these genes are known NF-κB target genes. We have identified one of the CD95-regulated genes as the serine/threonine kinase (SNF1/AMP kinase-related kinase (SNARK)), which is induced in response to various forms of metabolic stress. We demonstrate that up-regulation of SNARK in response to CD95 ligand and tumor necrosis factor α depends on activation of NF-κB. Overexpression of SNARK rendered tumor cells more resistant, whereas a kinase-inactive mutant of SNARK sensitized cells to CD95-mediated apoptosis. Furthermore, small interfering RNA-mediated knockdown of SNARK increased the sensitivity of tumor cells to CD95 ligand- and TRAIL-induced apoptosis. Importantly, cells with reduced expression of SNARK also showed reduced motility and invasiveness in response to CD95 engagement. SNARK therefore represents an NF-κB-regulated anti-apoptotic gene that contributes to the tumor-promoting activity of CD95 in apoptosis-resistant tumor cells.

CD95 (APO-1/Fas) is best known as a death receptor, inducing apoptosis in various tissues by recruiting initiator caspases to the death-inducing signaling complex leading to their activation (1, 2). It has been known for many years that CD95 can also activate nonapoptotic pathways such as the NF-κB, the mitogen-activated protein kinase pathways Erk1/2, JNK1/2, and p38, and the phosphatidylinositol 3-kinase/Akt pathway (3). Recently, it has become clear that activation of a combination of these pathways in apoptosis-resistant tumor cells can increase their motility and invasiveness (4), providing an explanation for experiments that identified CD95 as a tumorigenic factor when expressed on certain tumor cells grown *in vivo* (5). We recently identified a defined number of genes that were up-regulated in response to CD95 stimulation that could be involved in the nonapoptotic activities of CD95 (4). However, the nature of most of the genes that mediate the tumor-promoting activity of CD95 remains unknown.

Members of the AMP kinase family are serine/threonine kinases that play a role in tumorigenesis (6). This activity is believed to be due to their activation by various forms of metabolic stress such as glucose deprivation, a condition to be expected within solid tumors. Currently, five members of this family are known: AMK-α1, AMPK-α2[1] (7), MELK (8), SNARK (9, 10), and ARK5 (11). The fourth member of the family, SNARK, was originally cloned from a rat kidney library but was recently isolated from a human testis cDNA library after identification through BLAST searches (10). SNARK is activated by glucose starvation and under these conditions induces acute cell-cell detachment (10). We have recently identified human SNARK in a gene screen as one of only 17 genes that were up-regulated more than 2-fold in CD95-stimulated MCF7-Fas-Bcl-x_L(FB) cells (4).

We now show that SNARK is up-regulated by CD95 stimulation on the mRNA as well as the protein level and requires the activation of NF-κB. SNARK was found to have anti-apoptotic activities, as it protected cells from CD95- and TRAIL-induced apoptosis, and a kinase-dead mutant of SNARK sensitized cells to CD95-mediated apoptosis. Down-modulation of SNARK using SNARK-specific siRNAs demonstrated that SNARK is also involved in the CD95-induced increase in motility and invasiveness of MCF7-FB cells. SNARK is therefore one of the anti-apoptotic genes that is induced in apoptosis-resistant tumor cells triggered through CD95, which causes increased invasiveness of tumor cells, identifying SNARK as a possible target for cancer therapy.

EXPERIMENTAL PROCEDURES

Cell Lines and Reagents—MCF7-FB cells were cultured as described (13). The embryonic kidney cell line HEK293T was maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum (Sigma) containing penicillin (100 units/ml) and streptomycin (100 μg/ml). The breast tumor cell line MCF7, the cervical carcinoma cell line, HeLa, and the renal adenocarcinoma cell lines, CAKI-1 and ACHN, were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (Sigma) containing penicillin (100 units/ml) and streptomycin (100 μg/ml). For transfection, cells were seeded into a 6-well plate at 2×10^5/well, and 2 μg of DNA or 100 pmol of siRNA were transfected with LipofectAMINE 2000 (Invitrogen). HEK293T cells were transfected with the calcium phosphate method. The anti-CD95 monoclonal antibody, anti-APO-1,

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[1] The abbreviations used are: AMPK, AMP kinase; SNARK, SNF1/AMPK-related kinase; TNF, tumor necrosis factor; siRNA, small interfering RNA; siSNARK, small interfering SNARK; FB, Fas-Bcl-x_L; Z, benzyloxycarbonyl; fmk, fluoromethyl ketone; RT, reverse transcription; HA, hemagglutinin; GST, glutathione *S*-transferase; LzCD95L, leucine zipper-tagged CD95 ligand; uPA, urokinase plasminogen activator; huSNARK, human SNARK.

has been described previously (14). Leucine zipper-tagged CD95 ligand (LzCD95L) was generated as described previously (12). TNFα was purchased from Peprotech (Rocky Hill, NJ) and used at a concentration of 1000 units/ml. The caspase inhibitors Z-VAD-fmk and Z-IETD-fmk were purchased from Calbiochem. All other chemicals were of analytical grade and purchased from Sigma.

Semiquantitative RT-PCR and Real Time PCR—Total RNA was prepared from different stimuli-induced cells using TRIzol reagent (Invitrogen). An equal amount of total RNA (2 μg) isolated from cells at each of the stimulated conditions was reverse transcribed using SuperScript II reverse transcriptase (Invitrogen). For each PCR target, only the dilution that yielded visible products within the linear amplification range for the untreated cDNA was shown. 10^7 MCF7-FB cells were preincubated with or without 40 μM Z-IETD-fmk and then treated or untreated for 8 h with anti-APO-1 (1 μg/ml). Real time PCR was performed as described (4). Briefly, reverse-transcribed cDNA was made from 1 μg of each RNA sample and used for all subsequent real time PCR reactions.[2] Cycles were run on an ABI 7700, and SYBR® green was used for fluorescent detection of double-stranded DNA, according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). Normalization of the initial RNA composition was calculated from concurrent runs with primers specific for actin. Triplicates were performed with each primer set for each RNA sample. The mid-linear range was used to establish the threshold for each oligonucleotide set. After normalization and the calculation of differences within each time point experiment subset, all data from each time point were pooled to calculate differences.

SNARK Cloning, Plasmid Construction, Site-directed Mutagenesis, and siRNAs—Human *SNARK* was isolated from the IMAGE clone (ID: 5088037) (4) using the PCR primer set cggaattcatggagtcgctggttttcgcgcggcgc and gctctagatcaggtgagctttgagcagaccctcag. PCR product was digested by EcoRI/XbaI and subcloned in EcoRI/XbaI-opened pcDNA3. Influenza hemagglutinin (HA) sequence was added in the 3′ primer, and the EcoRI/XbaI-digested PCR product (huSNARK-HA) was subcloned into the EcoRI/XbaI-digested pcDNA3. Site-directed mutagenesis was performed with a QuikChange II kit (Stratagene, La Jolla, CA) to generate the kinase-dead SNARK by changing the Lys-82 to Arg using the pcDNA3-huSNARK wild-type vector as a template. The oligonucleotide used was gcgcctggtggccatcaggtcaatccggaaggac. All constructs were confirmed by sequencing. The control, Cys-3-bound luciferase siRNA, was purchased from Dharmacon (Lafayette). SNARK-targeting siRNA SMARTpool (SMART) was designed and produced by Dharmacon from the accession number NM_030952.1. We designed a second SNARK siRNA called siSNARK, which targets the sequence aacagaguuguccaccgagau in SNARK and is manufactured by Dharmacon.

Western Blotting and Antibodies—Cells were lysed for 30 min at 4 °C in lysis buffer (30 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture (Sigma)), and protein concentration was determined by Bradford assay (Bio-Rad). Lysates (100 μg) were resolved on 12% SDS-polyacrylamide gels and blotted with the indicated antibodies. Anti-caspase-8 (clone C-15) antibody was described previously (4), the anti-HA (clone HA.11) monoclonal antibody was purchased from Covance (Berkeley, CA), and the rabbit polyclonal anti-huSNARK antibody was generated by Zymed Laboratories Inc. (South San Francisco, CA) by immunizing rabbits with the SNARK sequence RQRESGYYSSPEPSES.

GST-SAMS Peptide—SAMS sequence (full sequence: HMRSAMSGLHLVKRR) was cloned in the pGEX-4T-1 vector. The construct was confirmed by sequencing. GST-SAMS was purified from BL21 *Escherichia coli* lysates with glutathione-Sepharose beads (Pierce) and eluted with buffer containing 50 mM Tris-HCl (pH 8) and 5 mM glutathione. Protein purity (>95%) and integrity were assessed by SDS gel electrophoresis followed by Coomassie Brilliant Blue staining.

Cell Death Assay—Cell viability was assessed using MTS assay according to the manufacturer's recommendations (Promega). In brief, 24 h after transfection, 2×10^4 cells were cultured for 16 h in flat-bottom, 96-well plates with the indicated concentrations of the apoptosis inducer in a final volume of 100 μl. 15 μl of MTS solution were added, and after 2–4 h of incubation at 37 °C, the absorbance was measured at 490 nm wavelength using a kinetic microplate reader (Molecular Devices).

Autophosphorylation and GST-SAMS Phosphorylation Assay—Immediately after stimulating MCF7-FB cells with LzCD95L or TNFα for 48 h after the transfection of 293T cells, cells were lysed for 30 min at 4 °C in lysis buffer supplemented with 10 mM NaF and 1 mM Na_3VO_4. Human SNARK was immunoprecipitated from 500 μg of protein containing lysate by incubation for 4 h at 4 °C with 10 μg/ml of either anti-huSNARK or anti-HA (clone HA.11) antibodies bound to protein A-Sepharose beads. Immune complexes were washed extensively in lysis buffer. An autophosphorylation assay was performed with these immunoprecipitates as described previously (9). Briefly, the immune complexes were incubated for 30 min at 30 °C in kinase buffer (15 mM HEPES (pH 7), 100 mM NaCl, 1% Triton X-100, 10% glycerol, 15 mM $MnCl_2$, 15 mM $MgCl_2$, 0.3 mM dithiothreitol) with 10 μCi of [γ-^{32}P]ATP, washed three times, resuspended in standard reducing sample buffer, boiled, and subjected to 8% SDS-PAGE. The gel was dried and exposed to film. pcDNA3 or pcDNA3-huSNARK-transfected 293T cell extracts (100 μg of protein) were used to determine the phosphorylation of the SAMS peptide (full sequence: HMRSAMSGLHLVKRR) fused to glutathione *S*-transferase. The lysates were incubated for 30 min at 30 °C in the presence of 5 μg of GST-SAMS (or control GST) in 50 μl of kinase buffer containing 10 μCi of [γ-^{32}P]ATP. The samples were then boiled in a standard reducing buffer and resolved on 12% SDS-PAGE. Autoradiography was performed on the dried gel.

Motility and Invasiveness Assay—These assays were performed as described previously (4). For invasion assays, Biocat Matrigel invasion chambers (BD Biosciences) containing 8-μm pore-size membranes were used. After rehydration of the matrigel, 75,000 cells were added to the top chamber in serum-free medium. The bottom chamber was filled with 10% serum-containing medium. LzCD95L or control supernatant was added to both chambers of each well. Cells were incubated for 72 h at 37 °C in a 5% CO_2 humidified incubator. For motility assays, Transwell insert chambers of 8 μm were used and the cells were treated and loaded as described above. To quantify invasion or motility, cells were removed from the top side of the membrane mechanically using a cotton-tipped swab, invading or migrating cells were fixed with methanol and stained with Giemsa stain, and a minimum of five representative fields were counted for each insert.

RESULTS AND DISCUSSION

SNARK Is a CD95-regulated Gene—We recently demonstrated that stimulating the death receptor CD95 does not induce apoptosis in multiple apoptosis-resistant tumor cells but instead triggers five apoptosis-independent pathways, three of which are important for increased motility and invasiveness of tumor cells in response to CD95 triggering (4). An Affymetrix gene chip analyses identified only 17 genes that were up-regulated more than 2-fold in CD95-stimulated MCF7-FB cells (4). Interestingly, 12 of these genes were known NF-κB target genes, which is consistent because NF-κB activation is required for the novel nonapoptotic function of CD95 as a tumor-promoting factor. One of these CD95-induced genes had an unknown open reading frame. After cloning this gene, we determined that it codes a 628-amino acid putative serine/threonine kinase. It was identical to the recently cloned human ortholog of the rat *SNARK* (10). *SNARK* is widely expressed in both normal tissues and tumor cells (9). Because we identified *SNARK* as part of a gene screen performed with CD95-stimulated cells, we first tested whether *SNARK* was indeed a CD95-inducible gene. Semiquantitative RT-PCR confirmed that SNARK was transcriptionally up-regulated in MCF7-FB cells as early as 30 min after stimulation with anti-APO-1 (Fig. 1*A*). Induction of SNARK mRNA was kinetically similar to induction of other genes that were identified in the gene chip analysis, such as *ICAM-1* and *A20*, which are bona fide NF-κB target genes. Induction of SNARK mRNA was also confirmed by real time PCR (Fig. 1*B*). To exclude *SNARK* as a CD95-induced target gene only in MCF7-FB cells that express large amounts of exogenous CD95, we tested MCF7 and HeLa cells, which are naturally CD95 apoptosis-resistant (Fig. 1*C*). In both cases, CD95 stimulation caused a marked induction of SNARK mRNA when cells were treated LzCD95L.

Up-regulation of SNARK in response to death receptor signaling was not limited to the mRNA level. The induction of endogenous SNARK protein was also detected in cells treated

[2] Specific primer sequences are available upon request.

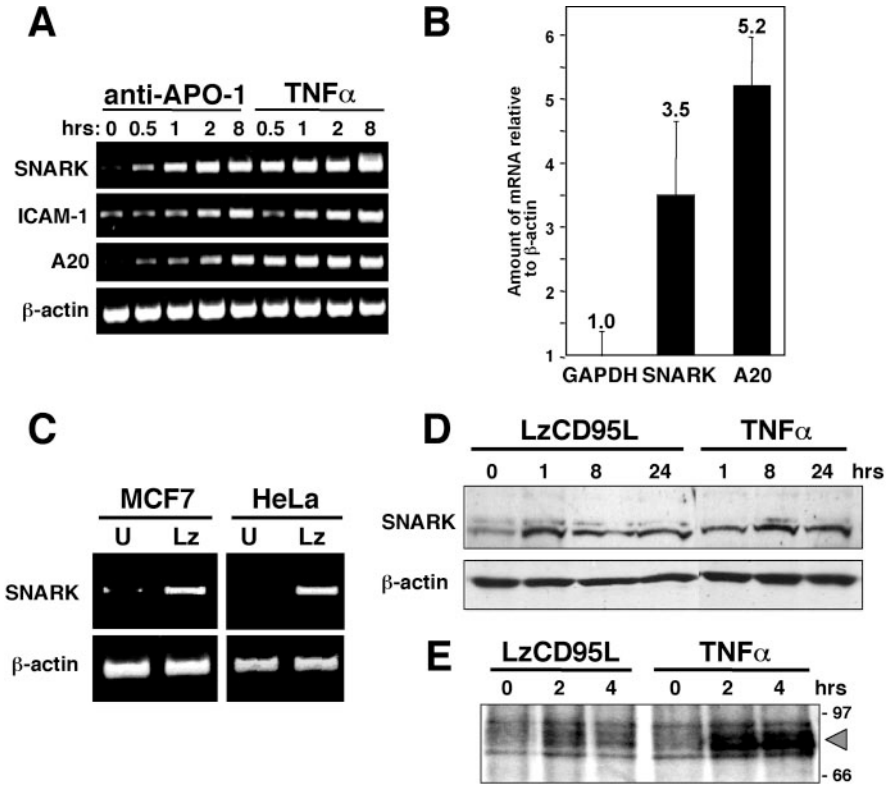

FIG. 1. **CD95 and TNF receptor-engagement induces up-regulation of huSNARK.** *A*, semiquantitative RT-PCR on RNA purified from MCF7-FB cells stimulated with anti-APO-1 or TNFα for the indicated times. *B*, MCF7-FB were treated with anti-APO-1 or left untreated for 8 h, and real time PCR was performed for SNARK. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) served as a second housekeeping gene control, and A20 served as a positive control. *C*, semiquantitative RT-PCR on RNA purified from MCF7 and HeLa cell lines treated (*Lz*) or untreated (*U*) for 2 h with LzCD95L. *D*, MCF7-FB cells were stimulated for the indicated times with either LzCD95L or TNFα. Cells were lysed, and a Western blot analysis was performed with the indicated antibodies. *E*, autophosphorylation kinase assay of immunoprecipitated SNARK from LzCD95L- and TNFα-stimulated MCF7-FB cells as described under "Experimental Procedures."

with LzCD95L or TNFα using an affinity-purified rabbit antibody we generated against a peptide located at the C terminus of SNARK in a region with high sequence diversity among different AMP kinase family members (Fig. 1*D*). This up-regulation of SNARK also caused a parallel increase in SNARK activity in cells, which was determined by performing an *in vitro* kinase assay on immunoprecipitated SNARK from LzCD95L- or TNFα-stimulated cells (Fig. 1*E*).

SNARK Is an NF-κB-regulated Gene—Induction of SNARK mRNA was direct because it could not be inhibited by treatment with the protein synthesis inhibitor cycloheximide (Fig. 2*A*). Also, the increase of SNARK mRNA was because of direct transcriptional activation rather than stabilization of mRNA because induction of *SNARK* could efficiently be blocked by actinomycin D (Fig. 2*A*). Interestingly, similar to TNFα and CD95 ligand treatment of MCF7-FB cells, TRAIL induced NF-κB activation in MCF7-FB cells (4) but only weakly induced SNARK mRNA (Fig. 2*B*).

Because 12 of 17 induced genes in the gene screen were known NF-κB-regulated genes, we tested whether inhibition of NF-κB would prevent up-regulation of *SNARK* (Fig. 2*C*). MCF7-FB cells were pretreated with the specific membrane-permeable NF-κB inhibitor, Tat-IκB^{mut}, described recently (4). CD95-induced up-regulation of *SNARK* was completely abolished by this reagent, confirming that activation of NF-κB is required for CD95-induced transcriptional activation of *SNARK*. This regulation was similar to a classical NF-κB target gene, *ICAM-1*. Finally, we found that the caspase-8 activity induced in the apoptosis-resistant MCF7-FB cells (13) does not play a significant role in the induction of *SNARK*; this is because preincubation of cells with the oligo caspase inhibitor Z-VAD-fmk did not inhibit up-regulation of SNARK mRNA (Fig. 2*D*, *upper panel*), although it blocked processing of caspase-8 in this experiment (Fig. 2*D*, *lower panel*). In summary, we have identified *SNARK* as a transcriptionally induced gene in CD95-stimulated cells. Its activation depends critically on activation of NF-κB, making it a potential mediator of the novel CD95 tumorigenic activities.

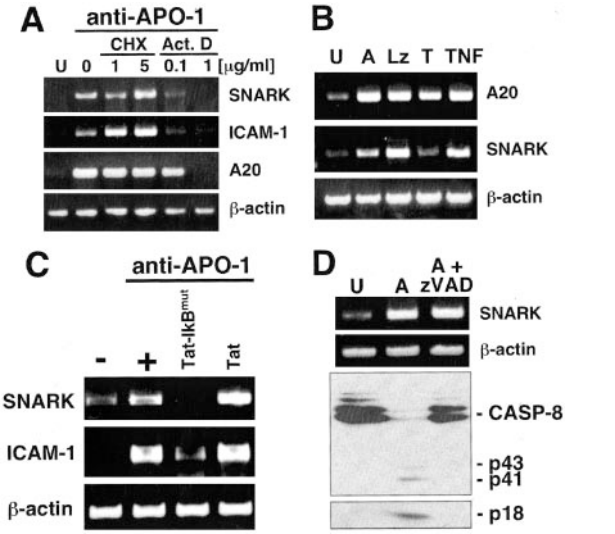

FIG. 2. **CD95-induced SNARK up-regulation is direct and relies on the NF-κB pathway but not on caspase activity.** *A*, semiquantitative RT-PCR was performed on the indicated genes with RNA from MCF7-FB cells preincubated with the indicated concentration of cycloheximide (*CHX*) or actinomycin D (*Act. D*) for 1 h and left unstimulated or stimulated for 2 h with anti-APO-1. *B*, RT-PCR of MCF7-FB cells treated for 8 h with either anti-APO-1 (*A*), LzCD95L (*Lz*), Lz-TRAIL (*T*), or TNFα (*TNF*) or left untreated (*U*). *C*, semiquantitative RT-PCR of MCF7-FB cells preincubated for 1 h with 10 μM GST-Tat (*Tat*) or GST-Tat-IκBα^{mut} (*Tat-IκBα^{mut}*) (see Ref. 4) and then stimulated for 8 h with 1 μg/ml anti-APO-1. *D*, MCF7-FB were preincubated for 30 min with Z-VAD-fmk (40 μM), left untreated (*U*), or treated for 4 h with anti-APO-1 (*A*). Semiquantitative RT-PCR on isolated RNA for the indicated genes (*top two panels*) and Western blot for caspase-8 on cell lysates (*bottom two panels*) were carried out.

Kinase-inactive SNARK Sensitizes Cells to CD95-mediated Apoptosis—We overexpressed the cloned human SNARK kinase in 293T cells and determined that it is an active kinase with typical autophosphorylation properties and with an apparent size of 70 kDa (Fig. 3*A*). In this experiment we also

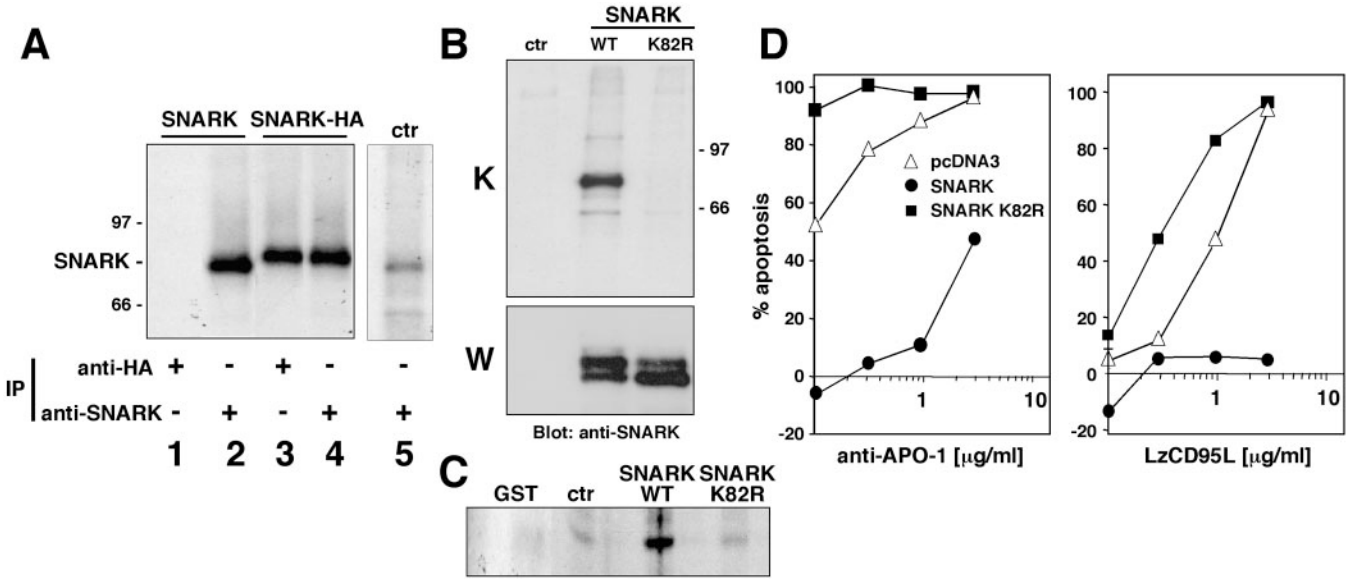

FIG. 3. **SNARK inhibits CD95-induced apoptosis.** *A*, *in vitro* kinase assay of SNARK or HA-SNARK immunoprecipitated (*IP*) with the indicated antibodies from lysates of transiently transfected 293T cells. *B*, 293T cells were transfected with wild-type or K82R mutant SNARK, and an autophosphorylation assay (*K*) was performed. Equal expression of SNARK and the mutant protein in 293T lysates was controlled by an anti-SNARK immunoblot (*W*). *C*, *in vitro* kinase assay was performed on the SAMS peptide. Lysates of empty vector (*ctr*), SNARK wild type, or K82R SNARK-transfected 293T cells were used to perform a kinase assay with GST-SAMS as substrate. The same amount of GST alone was incubated with the SNARK wild-type-transfected 293T lysate and used as control to demonstrate the specific phosphorylation of SAMS peptide. *D*, huSNARK inhibits CD95-induced apoptosis, and kinase-dead SNARK (SNARK K82R) sensitizes ACHN cells to CD95-induced apoptosis. ACHN cells were transfected with the indicated constructs and stimulated for 16 h with the indicated stimuli. The quantification of cell death was performed with an MTS assay in triplicates. Standard deviation is shown, but the error was too small to be visible in the graph.

detected a phosphorylated protein in control-transfected cells that comigrated with immunoprecipitated untagged SNARK, which was likely corresponding to active endogenous SNARK in these cells (Fig. 3*A*, *lane 5*).

To determine the function of endogenous SNARK in apoptosis, we generated a kinase-inactive mutant (Fig. 3*B*). This mutant was generated by replacing lysine 82, a highly conserved position in the AMP kinase family, with arginine, causing it to act in a dominant negative fashion when overexpressed (17, 18). The kinase function of the K82R mutant of SNARK was inactive, as it did not undergo autophosphorylation (Fig. 3*B*). It also did not phosphorylate a substrate comprising a fusion protein of GST with the SAMS peptide, which is the optimal sequence for phosphorylation by members of the AMP kinase family (19) (Fig. 3*C*). We then overexpressed either SNARK or SNARK K82R in the renal cell carcinoma cell line ACHN (12), which expressed endogenous SNARK (data not shown) and induced apoptosis with either anti-APO-1 or LzCD95L. ACHN cells were significantly protected by the expression of SNARK. This inhibition was dependent on the kinase activity of SNARK because SNARK K82R could not protect cells from CD95-mediated apoptosis. In contrast, SNARK K82R rendered cells even more susceptible to CD95-mediated apoptosis, suggesting that endogenous SNARK, which is induced upon triggering these cells through CD95 (data not shown), was inhibited in a dominant negative manner by the overexpressed mutant of SNARK. Transient overexpression of SNARK also protected another renal cell carcinoma cell line, CAKI-1 (12), from both CD95 ligand and TRAIL-induced apoptosis (data not shown). Taken together, our data suggested that SNARK is an NF-κB-regulated, CD95-inducible kinase that renders cells resistant to death receptor-mediated apoptosis. This activity is consistent with other CD95-induced genes that we recently found were up-regulated in CD95-stimulated, apoptosis-resistant cells (4). Many of these genes were anti-apoptotic and/or potentially tumorigenic.

SNARK Is an Anti-apoptotic Gene Required for CD95-induced Increase of Motility and Invasiveness of CD95 Apoptosis-resistant Tumor Cells—To directly determine the function of *SNARK* as a CD95-regulated gene, we reduced the expression of SNARK using siRNAs. Using RT-PCR, ACHN cells were found to express endogenous SNARK (Fig. 4*A*, *lane 1*); however, expression could be enhanced by treating cells with LzCD95L (Fig. 4, *lane 2*). Treating the cells with two independent siRNAs (siSNARK and an siRNA SMARTpool) for 24 h resulted in a significant reduction of SNARK mRNA (Fig. 4*A*, *lanes 3* and *4*). ACHN cells treated with either of the SNARK siRNAs rendered them more sensitive to CD95-mediated apoptosis (Fig. 4*B*). SNARK also inhibited TRAIL-induced apoptosis. ACHN cells treated with SNARK siRNA were significantly more sensitive to TRAIL-induced apoptosis (Fig. 4*C*). These experiments confirmed the anti-apoptotic activity of endogenous SNARK for death receptor-induced apoptosis. SNARK was one of only 17 CD95-induced genes in MCF7-FB cells, which respond to CD95 triggering with increased motility and invasiveness. We therefore tested whether the loss of SNARK would inhibit the CD95-mediated migration and invasiveness characteristics of these cells. As reported previously, MCF7-FB cells responded to stimulation with LzCD95L with up-regulation of typical NF-κB target genes such as urokinase plasminogen activator (*uPA*), *A20*, and *SNARK* (Fig. 4*D*, *lanes 1* and *2*). Treating the cells with either of the siRNAs directed at the SNARK mRNA resulted in a significantly reduced up-regulation of SNARK without affecting induction of uPA or A20 (Fig. 4*D*, *lanes 3* and *4*). We then subjected siRNA SNARK-treated MCF7-FB cells to motility and invasiveness assays, as described recently (4). Both CD95-induced motility and invasiveness were severely reduced in siSNARK-treated cells without affecting the general viability of these cells (data not shown), identifying *SNARK* as a gene that regulates this novel non-apoptotic activity of CD95.

Stress induced by treatment with AMP or glucose deprivation activates human and rat SNARK (9, 10). We incubated

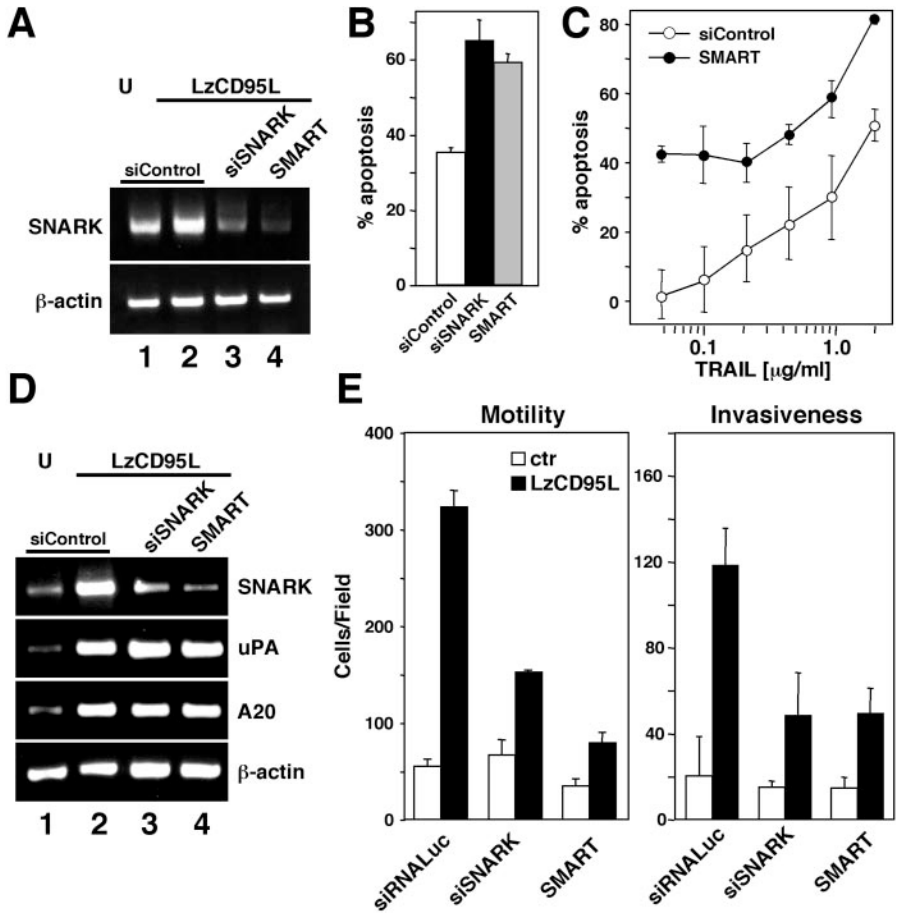

FIG. 4. **SNARK knock-down increases the sensitivity toward CD95 ligand and TRAIL-triggered apoptosis and inhibits CD95-induced motility and invasiveness.** *A*, semiquantitative RT-PCR of ACHN cells transfected with an siRNA control (siRNA luciferase-Cy3) or with two different SNARK-specific siRNAs for 24 h (see "Experimental Procedures"), left untreated (*U*), or treated for 2 h with LzCD95L. Cells were preincubated for 1 h with 40 μM Z-VAD-fmk. ACHN cells were transfected with siRNA control or siRNA SNARK and after 24 h were incubated for 16 h with 1 μg/ml LzCD95L (*B*) or the indicated concentrations of TRAIL (*C*). Cell death was quantified by an MTS assay. Experiments were performed in triplicates, and standard deviation is shown. *D*, semiquantitative RT-PCR of MCF7-FB cells transfected with siRNAs and treated with LzCD95L as in *A*. *E*, MCF7-FB cells were transfected with the indicated siRNA constructs and after 24 h were subjected to *in vitro* motility and invasiveness assays. *ctr*, control.

MCF7 cells with 200 μM AMP but did not observe activation of SNARK (data not shown). Both AMPK and another member of the AMPK family, ARK5, which shows 55% overall homology with SNARK including 84% identity within the N-terminal kinase domain, were recently described as having anti-apoptotic activity (11, 16). Overexpression of ARK5 rendered cells more resistant to TNFα, TRAIL, and glucose deprivation. It was therefore postulated that ARK5 could promote tumor cell survival during nutrient starvation. The recent recognition that CD95 has a tumorigenic activity on tumor cells both *in vitro* (4) as well as *in vivo* (5) suggests the existence of protumorigenic genes that are induced in response to CD95 stimulation. *SNARK* induction requires activation of NF-κB, which is critical for the CD95 protumorigenic activities (4). We recently demonstrated that *uPA*, one of the NF-κB-regulated genes induced by CD95, is critical for the CD95-induced motility and invasiveness of apoptosis-resistant tumor cells. Blocking the activity of uPA blocked the ability of cells to invade in response to CD95 stimulation (4). *SNARK* is the second of the recently identified CD95-induced genes tested, and siRNA-induced down-modulation of SNARK demonstrated that SNARK, too, is important for the novel CD95-dependent tumorigenic activities.

Knocking down SNARK seemed to render cells more sensitive to TRAIL-induced apoptosis then apoptosis triggered through CD95. Although *SNARK* is induced by CD95 stimulation, it may not play its role as an anti-apoptotic factor predominantly in CD95-mediated apoptosis. CD95 apoptosis resistance can be achieved in many ways by tumor cells.[3] Our data suggest that stimulating CD95 on tumor cells that are resistant to CD95-mediated apoptosis with whatever mechanism can up-regulate tumorigenic genes, such as *SNARK*, may give these cells further protection to CD95-mediated apoptosis. This stimulation can also give tumor cells resistance to other apoptosis inducers (*e.g.* TRAIL) and induce tumorigenic pathways that can cause increased motility and invasiveness.

Reducing the expression of SNARK did not affect the expression of uPA, suggesting that SNARK is not required for the induction of uPA. The fact that CD95 activates at least five different nonapoptotic signaling pathways independently (4), resulting in the induction of multiple genes, suggests that the regulation of these activities is complex. Future work will have to determine how many genes and pathways are involved in the regulation of these activities, ultimately giving a better understanding of the nonapoptotic functions of CD95 in cancer cells.

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The role of receptor internalization in CD95 signaling

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Activation of the cell surface CD95 receptor triggers a cascade of signaling events, including assembly of the death-inducing signaling complex (DISC), that culminate in cellular apoptosis. In this study, we demonstrate a general requirement of receptor internalization for CD95 ligand-mediated DISC amplification, caspase activation and apoptosis in type I cells. Recruitment of DISC components to the activated receptor predominantly occurs after the receptor has moved into an endosomal compartment and blockade of CD95 internalization impairs DISC formation and apoptosis. In contrast, CD95 ligand stimulation of cells unable to internalize CD95 results in activation of proliferative Erk and NF-κB signaling pathways. Hence, the subcellular localization and internalization pathways of CD95 play important roles in controlling activation of distinct signaling cascades to determine divergent cellular fates.

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Introduction

Surface receptors transduce signals derived from the extracellular milieu to evoke a diverse range of cellular responses. This process is initiated upon ligand binding and transduced through the spatial and temporal regulation of physical interactions of receptors with intracellular signaling molecules. Gain- or loss-of function mutants alter the normal balance of cellular homeostasis that, in turn, can induce oncogenesis and/or developmental arrest. For many receptors, triggering by ligand results in receptor clustering that is followed by downregulation of activated surface receptors through endocytosis and subsequent lysosomal degradation (Ceresa and Schmid, 2000). These latter steps typically attenuate signaling via removal of activated receptor complexes. Recent studies, however, indicate that receptor internalization can target activated receptors to the endocytic compartment, and contributes to both the intensity of signaling and assembly of signaling complexes (Miaczynska *et al*, 2004b).

CD95 (CD95/APO-1/TNFRSF6) is a prototypic death receptor belonging to the tumor necrosis factor (TNF) receptor superfamily (Li-Weber and Krammer, 2003). Interactions of CD95 with its ligand, CD95L (CD178/FasL/TNSF6), play a pivotal role in the regulation of peripheral tolerance and lymphoid homeostasis. Natural mutations within CD95 and CD95L in humans and mice are associated with the development of autoimmune lymphoproliferative syndromes (Nagata, 1999). CD95 is expressed on the surface of cells as preassociated homotrimers and, upon CD95L binding, undergoes a conformational change to reveal its cytoplasmic death domain (DD) to favor homotypic interactions with other DD-containing proteins (Itoh and Nagata, 1993; Boldin *et al*, 1995; Chinnaiyan *et al*, 1995; Siegel *et al*, 2000). Additional interactions mediated through the N-terminal 'death effector domain' (DED) of FADD with DED domains encoded within procaspase-8 and -10 assemble the death-inducing signaling complex (DISC) (Peter and Krammer, 2003). Efficient DISC assembly provides a molecular scaffold concentrating cysteine proteases to induce autoproteolytic cleavage of caspase-8 and, in turn, subsequent activation of the apoptotic pathway.

CD95-mediated apoptosis is transduced through two general modes (Algeciras-Schimnich *et al*, 2003; Barnhart *et al*, 2003). Type I cells exhibit rapid receptor internalization and form large amounts of DISC, while type II cells are more dependent upon the mitochondrial amplification pathway and exhibit quantitatively less and slower DISC assembly. We demonstrate here that CD95 internalization in type I cells plays a previously unrecognized requisite role in CD95L-induced activation of apoptotic pathways. In contrast, engagement of CD95 without receptor internalization results in activation of nonapoptotic signaling pathways. Hence, the subcellular compartment of CD95 signaling activates divergent biochemical pathways to promote distinct survival or apoptotic cellular fates.

Results

Expression of a plasma membrane localized PIP_2-specific 5′-phosphatase modulates PIP_2 levels and inhibits CD95L-induced CD95 internalization and apoptosis

We have previously demonstrated that disruption of filamentous actin inhibits CD95 internalization, a process that normally proceeds through a clathrin-mediated endocytic compartment, and renders cells more resistant to CD95-mediated apoptosis (Algeciras-Schimnich *et al*, 2002; Algeciras-Schimnich and Peter, 2003). As cellular levels of PIP_2 ($PtdIns(4,5)P_2$) have been shown to regulate clathrin-

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mediated endocytosis (Martin, 2001), we employed an enzymatic approach using the *Saccharomyces cerevisiae Inp54* 5-phosphatase (INP54p) that hydrolyzes PIP_2 to PI(4)P (Stolz *et al*, 1998). Targeting of a green fluorescent protein (GFP)–INP54p fusion protein to the plasma membrane (PM) was achieved by attaching a myristoylation/palmitoylation sequence from the Fyn protein tyrosine kinase (Shenoy-Scaria *et al*, 1993; Raucher *et al*, 2000) (Supplementary Figure 1A, middle panels). Expression of FynC-GFP-INP54p reduced PIP_2 levels in >98% of transfected GFP^+, but not of GFP^-, cells (Supplementary Figure 1B, middle panel). In contrast, expression of a mutant in which Cys 3 and 6, important for palmitoylation and PM localization, were mutated to Ser (designated as FynS-GFP-INP54p) resulted in a cytoplasmic distribution (Supplementary Figure 1A, right panels) and lesser effects on PIP_2 levels (Supplementary Figure 1B, bottom panel). PIP_2 levels were unaffected in cells expressing a control FynC-GFP cDNA (top panel).

We next investigated the functional consequences of reduced PIP_2 in CD95 function. BJAB cells, transiently transfected with FynC-GFP-INP54p, FynS-GFP-INP54p or FynC-GFP, were stimulated with Flag-tagged (Flag-)CD95L (SuperFasL, Apotech) and the degree of apoptosis was assessed by TUNEL staining. In FynC-GFP^+ or FynS-GFP-$INP54p^+$ cells, ~65% of cells were $TUNEL^+$ following CD95L stimulation (Supplementary Figure 1C, left and right panels). In contrast, <5% of FynC-GFP-$INP54p^+$ BJAB cells were $TUNEL^+$ in response to CD95L treatment (middle panel). Inhibition of CD95L-induced apoptosis was observed at 5, 16, 24 and 48 h (Supplementary Figure 1D). Similar inhibition of CD95L-induced apoptosis was also observed in type I SKW6.4 cells (Supplementary Figure 2A). In contrast, expression of FynC-GFP-INP54p did not affect CD95L-mediated apoptosis in type II Jurkat T cells (Supplementary Figure 2B). FynC-GFP-INP54p expression did not induce a general defect in the apoptotic machinery in BJAB cells, as etoposide-induced apoptosis, which acts through a mitochondria-mediated intrinsic pathway (Shimizu *et al*, 1996), was unaffected (Supplementary Figure 2C). In addition, apoptosis-resistant FynC-GFP-$INP54p^+$ cells, following CD95L activation, retained normal cellular growth and were grossly indistinguishable from normal BJAB cells (data not shown).

To define the biochemical basis by which the CD95-mediated apoptosis was affected by FynC-GFP-INP54p, BJAB cells were stimulated with Flag-CD95L and activation of caspase-8 and -3 measured by immunoblotting for their self-cleavage products. While treatment of BJAB cells with Flag-CD95L resulted in a time dependent cleavage of both caspases (Figure 1A, lanes 1–4), no evidence for caspases-8 or -3 activation was detected in FynC-GFP-$INP54p^+$ BJAB cells (lanes 5–8). We next analyzed the recruitment of FADD to activated CD95, one of the most proximal biochemical events in DISC formation. While FADD readily co-immunoprecipitated with CD95L-bound CD95 in control cells, no association of FADD with activated CD95 was detected in FynC-GFP-$INP54p^+$ BJAB cells (Figure 1B, top row). Similarly, while caspase-8 and -10 were readily co-immunoprecipitated with CD95/CD95L complexes in control cells, neither was detected with activated CD95 in FynC-$INP54p^+$ BJAB cells (second and third rows). Taken together, these observations indicate that FynC-GFP-INP54p reduces cellular PIP_2 levels as well as CD95L-induced FADD association with activated CD95, caspase activation and apoptosis.

As CD95L binding to CD95 was not altered by FynC-GFP-INP54p (Supplementary Figure 3A), we assessed the effects of FynC-GFP-INP54p on CD95 clustering. Cells were incubated with Flag-CD95L at 4°C, activation was induced by incubation at 37°C, and localization of CD95 analyzed by deconvolution microscopy. In wt BJAB cells (GFP^- cells), CD95L induced small 'patch-like' receptor clusters at the PM within 5 min after stimulation (Figure 1C, panels 4–6, left cell). Expression of FynC-GFP-INP54p did not affect the ability of CD95L to induce CD95 clustering at the PM 5 min following activation (Figure 1C, panels 4–6, GFP^+ cells). By 15 and 30 min, the clustered CD95 in GFP^- cells had internalized to intracellular compartments (Figure 1C, panels 7–12, left cell). In contrast, CD95 remained clustered at the PM for at least 30 min in FynC-GFP-$INP54p^+$ cells without any significant internalization following CD95L stimulation (Figure 1C, panels 7–12, right cell).

These results were further supported by flow-cytometric studies. In BJAB cells transfected with control FynC-GFP, CD95 downregulation was detected within 15 min following CD95L activation (Figure 1D, top panel). In contrast, FynC-GFP-$INP54p^+$ cells were unable to downregulate surface CD95 even 30 min following CD95L activation (bottom panel). Similar results were found for SKW6.4 cells (Supplementary Figure 2D).

Membrane-bound CD95L (mCD95L)-induced apoptosis requires CD95 internalization

Our data so far suggested that CD95-mediated apoptosis in response to a soluble form of CD95L (sCD95L) stimulation requires receptor internalization. However, it is widely assumed that the physiologic stimulus for CD95 is more likely to be mCD95L. We therefore incubated murine CT26 cells expressing human mCD95L (CT26mCD95L) with SKW6.4 cells. No soluble CD95L could be detected in CT26mCD95L cells or concentrated supernatant derived from cultures of these cells (Supplementary Figure 4B and data not shown). In SKW6.4 cells, CD95 was efficiently internalized when co-incubated with CT26mCD95L, but not in untreated SKW6.4 cells (Figure 2A and Supplementary Figure 4A and B). Correspondingly, CT26mCD95L cells induced a time-dependent processing of procaspase-8 (Supplementary Figure 4C). When SKW6.4 cells were overlaid on adherent CT26 cells, CD95 internalized in SKW6.4 cells contacting CT26mCD95L, but not control CT26, cells (Figure 2B, bottom panels). These data suggest that mCD95L induces internalization of CD95 as much as sCD95L.

We have previously shown that unmodified sCD95L does not induce CD95 apoptosis in type I cells (Algeciras-Schimnich *et al*, 2003). However, to exclude that the internalization and apoptosis observed in cells exposed to mCD95L were not due to very small amounts of secreted sCD95L, we expressed a mutant form of human mCD95L (designated as DA4) that cannot be cleaved from the membrane surface (Tanaka *et al*, 1998) on the surface of chicken DT40 B cells (Supplementary Figure 3B). Deconvolution microscopy confirmed that CD95 internalized into the cytoplasmic compartment when incubated with mCD95L(DA4)-expressing DT40 cells (Figure 2C, right panel), but not when incubated with control DT40 cells (left panel).

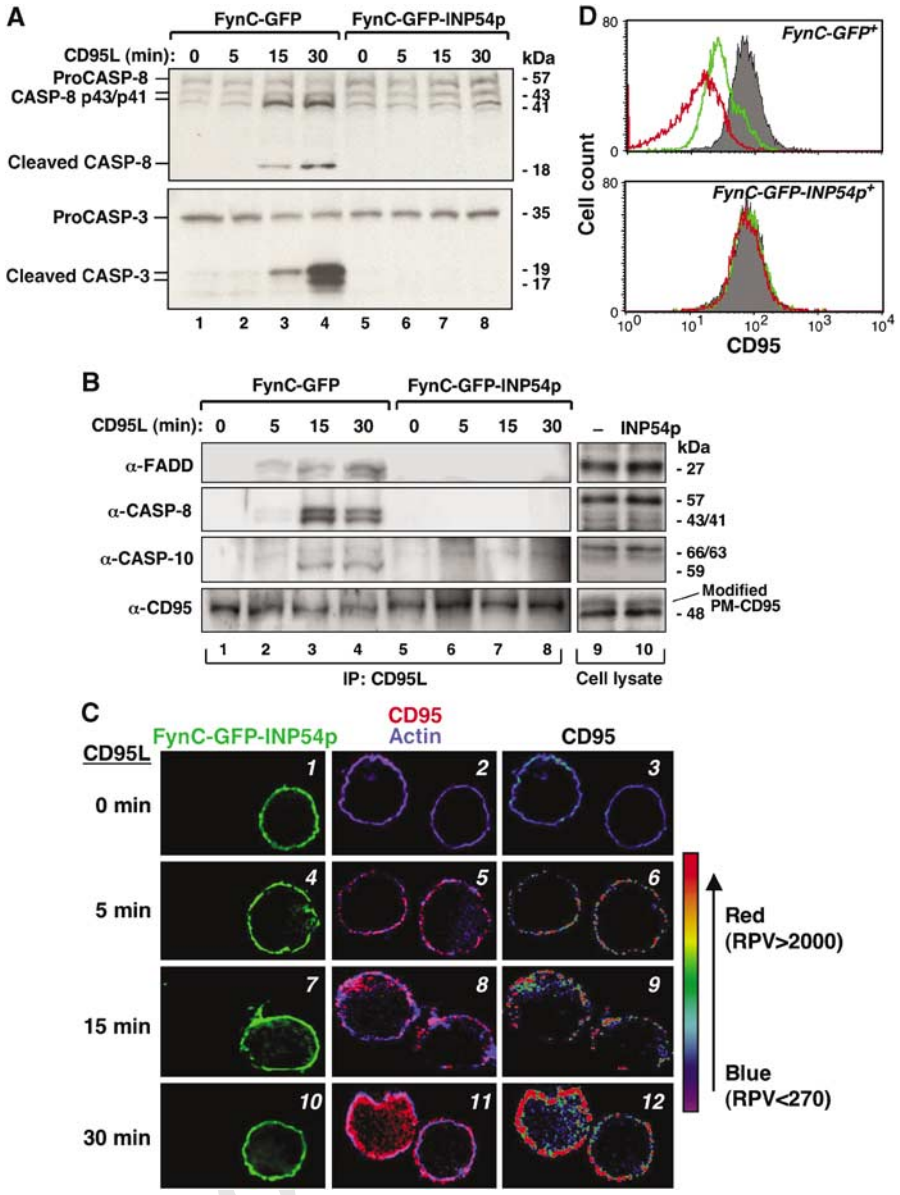

Figure 1 Defective CD95 internalization and apoptosis induction in FynC-GFP-INP54p-expressing cells. (**A**) Defective caspase activation in PM-targeted INP54p-expressing cells. BJAB cells expressing a control vector (lanes 1–4) or FynC-GFP-INP54p (lanes 5–8) were stimulated with Flag-CD95L for the indicated times. Cells were lysed and analyzed for caspase-8 (top) and -3 (bottom) cleavage. Transfection efficiency was >90% using a dual-transfection protocol. Data shown are representative of two experiments. (**B**) Defective DISC assembly in PM-targeted INP54p-expressing cells. BJAB cells were treated as described in (A). CD95L–CD95 complexes were immunoprecipitated by use of anti-Flag Ab-coupled beads and analyzed for associated DISC proteins (lanes 1–8). Cell lysates were also analyzed for DISC proteins and CD95 expression. Data shown are representative of three experiments. (**C**) BJAB cells transiently transfected with FynC-GFP-INP54p were stimulated with Flag-CD95L for the indicated times. Permeabilized cells were visualized by deconvolution microscopy for GFP (green) in the left panels and stained for CD95 (red) and F-actin (blue) in the middle panels. Quantitative image analysis with RPVs recorded for CD95 fluorescence signals is shown in the right panels. Red indicates the highest and blue represents the lowest fluorescence intensity. RPV = relative pixel value. Data shown are representative of >150 cells analyzed. (**D**) Inhibition of CD95 internalization by FynC-GFP-INP54p. BJAB cells, transfected with FynC-GFP (left) or FynC-GFP-INP54p (right), were incubated with Flag-CD95L at 37°C for 15 (green) or 30 mins (red). As a control, cells were incubated with Flag-CD95L on ice (0 min, black). The remaining surface CD95 was detected by staining with an anti-CD95 mAb (DX2) and analyzed by flow cytometry. Data shown are representative of four experiments.

We next tested whether apoptosis induced by mCD95L was affected by FynC-GFP-INP54p expression. While DT40 cells expressing the CD95L(DA4) mutant induced apoptosis in FynC-GFP$^+$ BJAB cells (Figure 2D, left panels), apoptosis was significantly attenuated at 16 and 24 h following engagement of CD95L(DA4) in FynC-GFP-INP54p$^+$ BJAB cells (right panels). Concurrently, surface CD95 was downregulated in FynC-GFP$^+$ BJAB cells at 30 and 60 min, but this was significantly compromised in FynC-GFP-INP54p$^+$ BJAB cells (Figure 2E).

Expression of FynC-GFP-INP54p in BJAB cells inhibited CD95L-induced apoptosis irrespective of the degree of CD95 oligomerization. Expression of FynC-GFP-INP54p inhibited apoptosis and CD95 downregulation induced via soluble and plate-bound crosslinked Flag-CD95L or anti CD95 mAb (CH-11) (Supplementary Figure 5A). Finally, CD95 activation of

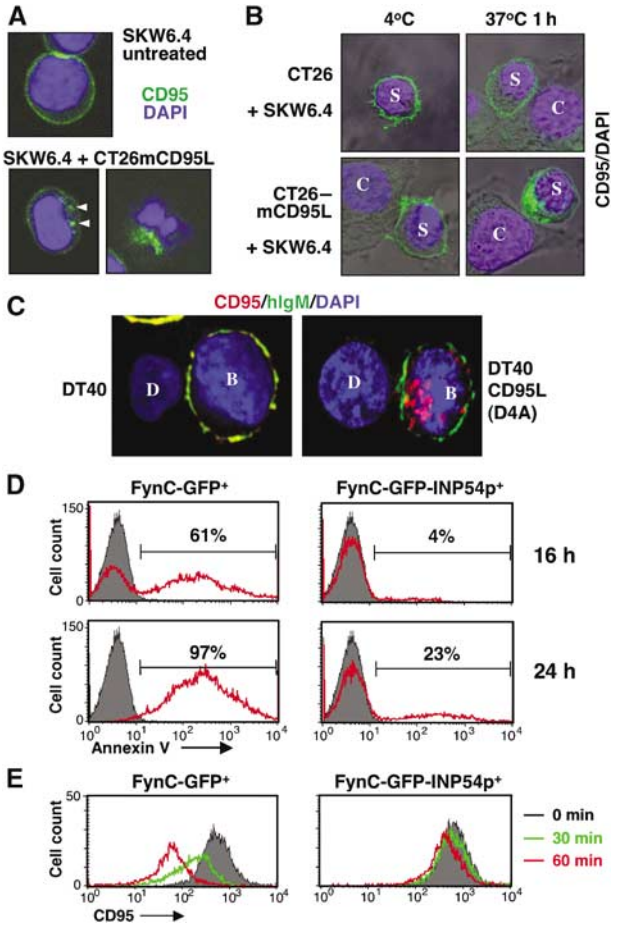

Figure 2 Membrane-bound CD95L induces internalization of CD95. (**A**) Internalization of CD95 on SKW6.4 cells following activation by mCD95L. SKW6.4 cells were incubated with FITC-DX2 on ice. Cells were then left untreated or incubated with detached CT26 cells expressing human mCD95L for 1 h. Nuclei were stained with DAPI and CD95 was visualized by confocal microscopy. The left bottom panel represents an early stage with intact nucleus and internalized CD95-containing vesicles (arrowheads). The right bottom panel shows a more advanced stage of apoptosis with nuclear fragmentation. (**B**) CD95 on SKW6.4 cells internalizes at the contact side with mCD95L-expressing CT26 cells. SKW6.4 cells (labeled S) with a bound biotin-labeled anti-CD95 mAb were plated on top of adherent CT26 or CT26mCD95L cells (labeled C) and incubated as indicated. CD95 was visualized by staining with streptavidin Alexa Flour 488. Nuclei were visualized by DAPI staining. (**C**) Internalization of CD95 on BJAB cells following activation by cleavage-resistant mCD95L(DA4). BJAB cells (labeled B) were incubated with chicken DT40 cells (labeled D) (left panel) or DT40 cells expressing human mCD95L(DA4) (right panel) for 1 h and then analyzed by deconvolution microscopy for CD95 (red), surface hIgM (green) and DAPI staining (blue). (**D**) Inhibition of mCD95L-induced apoptosis in FynC-GFP-INP54p-expressing cells. BJAB cells were transfected with FynC-GFP-INP54p or control FynC-GFP and incubated with DT40 cells expressing a noncleavable mutant of human mCD95L(DA4) at a ratio of 1:5 (BJAB:DT40) for the indicated times. Apoptosis of GFP^+ cells was assessed by staining with Annexin V. Data shown are representative of two independent experiments. (**E**) Inhibition of mCD95L-mediated CD95 downregulation in FynC-GFP-INP54p-expressing cells. BJAB cells, transfected as described in D, were incubated with DT40 cells expressing mCD95L(DA4) for 30 or 60 min. Surface CD95 expression on GFP^+ cells was assessed by flow cytometry. Data shown are representative of two independent experiments.

BJAB cells using beads covalently coupled with an anti-CD95 mAb or Flag-CD95L was also inhibited by expression of FynC-GFP-INP54p (Supplementary Figure 5B and data not shown). In contrast to the agonistic anti-CD95 mAbs, treatment of H9 cells with an antagonistic anti-CD95 mAb (ZB4) failed to induce CD95 internalization (Supplementary Figure 5C). Hence, independent of the methodology of stimulation, expression of FynC-GFP-INP54p inhibits CD95 internalization and apoptosis and apoptosis is dependent on internalization of CD95 in cells treated with sCD95L or mCD95L.

Clathrin-mediated endocytosis is required for CD95-induced apoptosis

Our data suggested that modulation of PIP_2 levels through INP54p rendered cells resistant to CD95-mediated apoptosis by blocking internalization of CD95. However, modulation of PIP_2 could result in global cellular changes that could cause cells to become resistant to CD95-mediated apoptosis by mechanisms other than receptor internalization. CD95 has been suggested to internalize through a clathrin-mediated endocytic pathway (Algeciras-Schimnich *et al*, 2002). To specifically interfere with this form of receptor internalization, we targeted expression of the AP-2 adaptor complex and clathrin heavy chain (CHC) proteins using RNA interference. Transfection of siRNAs specific for CHC or AP-2($\alpha \pm \mu_2$) adaptor subunits resulted in significant reduction in their levels of protein expression (Figure 3A, lanes 2–6) (Motley *et al*, 2003).

Correspondingly, knockdown of AP-2 (α or μ_2) alone resulted in a moderate decrease in CD95L-induced downregulation of surface CD95 (Figure 3B, panels 2 and 3). Knockdown of both AP-2 (α and μ_2) subunits or CHC resulted in a greater compromise in CD95 downregulation (panels 4 and 5). Finally, knockdown of both AP-2($\alpha + \mu_2$) and CHC resulted in the greatest inhibition of CD95 downregulation, though the basal level of surface CD95 expression was also decreased (panel 6).

The degree of compromise observed in CD95 downregulation directly correlated with the degree of apoptosis induced by CD95L. Gene knockdown of either AP-2($\alpha + \mu_2$) or CHC resulted in ~50–80% decrease in CD95L-induced apoptosis, respectively, and the combination of AP-2($\alpha + \mu_2$) and CHC siRNAs, which demonstrated the greatest inhibition in CD95L-induced CD95 downregulation, resulted in total protection from CD95L-induced apoptosis (Figure 3C).

Interestingly, the inducible association of FADD with CD95 was compromised in cells transfected with AP-2($\alpha + \mu_2$) or CHC siRNAs (Figure 3D, lanes 3 and 4). The lack of FADD association severely reduced the formation of the DISC as neither FADD nor caspase-8 co-immunoprecipitated with CD95 at 5, 15 or 30 min following CD95 activation in cells transfected with CHC siRNAs (Figure 3E). These results suggest a role of receptor internalization in assembly of the DISC.

Finally, we analyzed the role of CD95 internalization in CD95L-induced apoptosis with peripheral human T lymphocytes (PBTs). PBTs were activated through CD3/CD28 and then transfected with siRNAs for AP-2($\alpha + \mu_2$) and GFP, the latter of which was utilized to monitor expression. GFP^{hi} cells were purified by cell sorting and analyzed for CD95 internalization and apoptosis. Transfection of AP-2($\alpha + \mu_2$) siRNAs in PBTs resulted in inhibition of CD95 downregula-

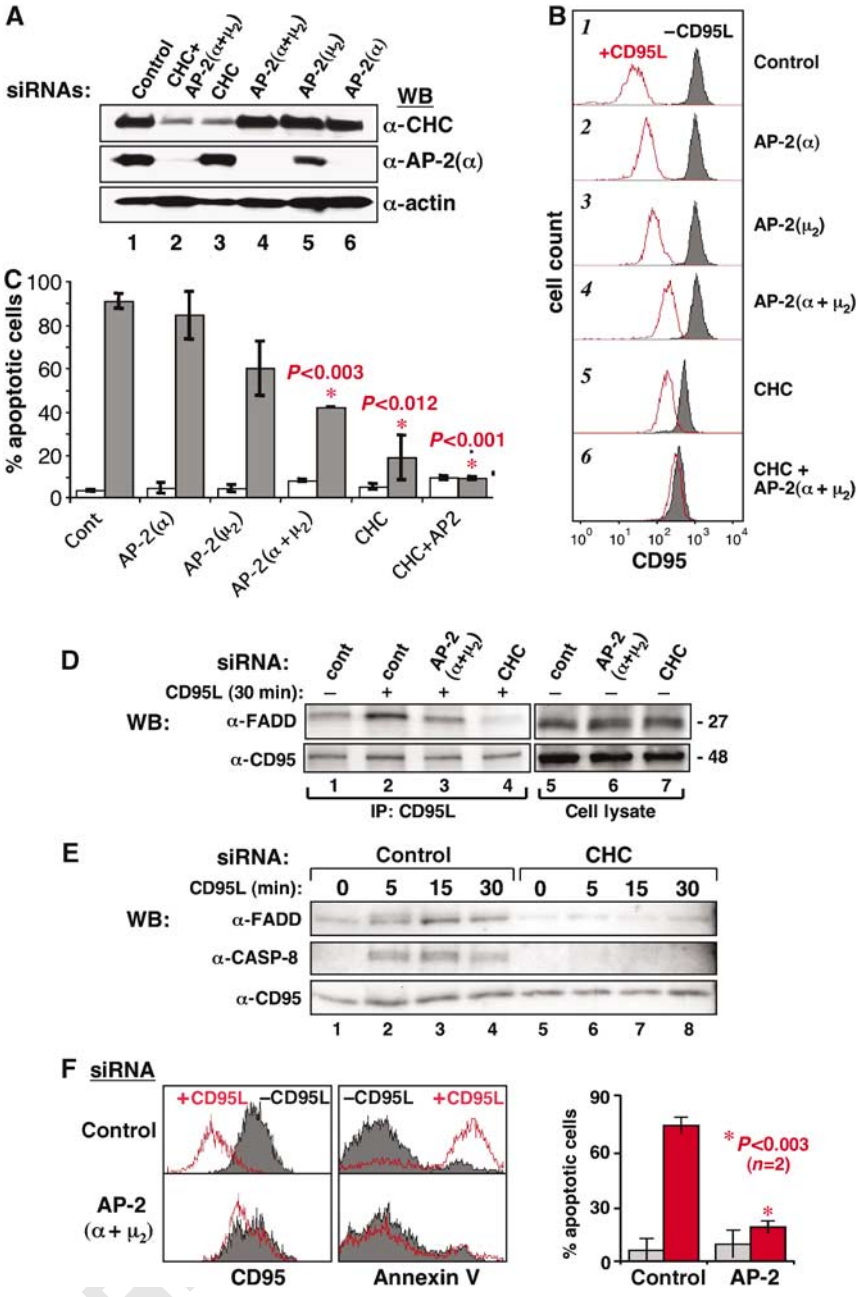

Figure 3 Clathrin-mediated endocytosis is required for CD95 downregulation and apoptosis. (**A**) Knockdown of CHC, AP-2 μ_2 and AP-2 α using siRNAs in BJAB cells. Efficiency of knockdown was monitored by blotting for the indicated proteins. Blotting for actin was used as a control for protein loading (bottom). (**B**) BJAB cells transfected with the indicated siRNAs, as described in (A), were incubated with Flag-CD95L for 30 min and surface CD95 expression assessed by flow cytometry. Red histograms indicate cells stimulated for 30 min, while the gray shadowed areas indicate basal levels of CD95 without CD95L stimulation. Data shown are representative of three experiments. (**C**) BJAB cells, as described in (A) and (B), were incubated in the presence (grey) or absence (white) of Flag-CD95L for 16 h and apoptotic cells quantified by staining with Annexin V and 7-AAD. Data shown are representative of threeriments. (**D**) CD95L-induced association of FADD with CD95 is inhibited by AP-2 and CHC siRNAs. Cells were transfected with control (lanes 1 and 2), AP-2 (lane 3) or CHC (lane 4) siRNAs, activated with Flag-CD95L for 30 min and FADD association with activated CD95 assessed by immunoprecipitating CD95L–CD95 complexes. Lanes 5–7 demonstrate comparable levels of FADD and CD95 in all cells. (**E**) FADD and caspase-8 association with CD95 is inhibited by CHC siRNAs. BJAB cells were transfected with control (lanes 1–4) and CHC (lanes 5–8) siRNAs and activated with Flag-CD95L for the indicated times. Association of FADD and caspase-8 with activated CD95 was assessed by immunoprecipitating for CD95L and immunoblotting for FADD, caspase-8 and CD95. (**F**) Clathrin-mediated endocytosis is required for CD95-mediated apoptosis in PBTs. Activated human $CD4^+$ PBTs were transfected with siRNAs for AP-2($\alpha + \mu_2$) and a GFP cDNA to monitor expression efficiency. Sorted GFP^{hi} cells were analyzed for CD95 downregulation (left) and apoptosis (right) following Flag-CD95L activation (30 min and 6 h, respectively). Red histograms indicate cells stimulated with CD95L while grey shadowed areas represent untreated cells. % apoptotic cells are quantified on the right. Data shown are representative of two experiments.

tion following CD95L activation (Figure 3F, left bottom panel). Correspondingly, these cells demonstrated compromised CD95L-induced apoptosis (Figure 3F, right bottom panel). In summary, our data indicated that inhibition of CD95 internalization in type I cells as well as in primary T

lymphocytes attenuated recruitment of DISC components to CD95 receptors and apoptosis.

Recruitment of DISC components following CD95 internalization

To directly follow the recruitment of DISC components to activated CD95 and to compare receptor signaling between type I and type II cells, we made use of a novel method to isolate receptor-containing internalized vesicles that has been used to detect internalizing TNF receptor and its signaling components (Schneider-Brachert *et al*, 2004). In this method, cells were incubated with biotinylated anti-CD95 (anti-APO-1) mAb, followed by addition of streptavidin coupled magnetic nanoparticles. Following internalization, cells were homogenized and magnetic vesicles isolated in a free flow apparatus employing a high-gradient magnetic field. Western blotting of receptor-containing vesicles for endosomal and lysosomal markers was performed to assess the different endocytic maturation stages of receptor-containing vesicles.

Consistent with the ability of CD95 to internalize in type I SKW6.4 cells, Rab4 and EEA-1, markers for endosomal trafficking, were readily detected within CD95-containing vesicles very early after stimulation and peaking at 10 min (Figure 4A). Already detectable at 3 min and peaking at 30 min, CD95-containing vesicles also had lysosomal characteristics as evidenced by the appearance of cathepsin D (CatD), suggesting rapid association/fusion of CD95-containing receptosomes with CatD-containing lysosomal compartments. While a low level of FADD was detected in CD95 containing membrane structures at basal levels, its appearance in magnetic vesicles peaked at 30 min. Similar to FADD, caspase-8 and its intermediate cleavage products peaked at 10 min and could be detected as late as 3 h following stimulation (data not shown), and suggested that most of the caspase-8 activation occurred while inside the cells located on endosomal and even lysosomal vesicles. A similar kinetics for association of caspase-10 within the CD95-containing vesicles was also observed.

In contrast to type I SKW6.4 cells, no significant increase in Rab4, EEA-1 or CatD was observed in type II Jurkat cells, suggesting a lack of directional movement of CD95 into endosomal vesicles (Figure 4B). Consistent with the delayed and lower amounts of DISC component assembly in type II cells, FADD, caspase-8 and -10 were detected at lower levels and at later time points than in type I cells.

Since differences observed between type I SKW6.4 and II Jurkat cells might be due to the lower levels of CD95 expression on type II cells or limited to only lymphoid cells (Huang *et al*, 2000), we analyzed type I ACHN cells that express lower levels of CD95 than type II HCT15 cells (Algeciras-Schimnich *et al*, 2003). Rab4 was detected within CD95-containing vesicles with maximal association at 5 min in ACHN cells, indicating that receptor internalization had already begun (Figure 4C). EEA-1 appeared at 5 min and peaked at 30 min, consistent with maturation to endosomal vesicles. CatD was also detected at 5 min with further increases to 60 min indicative of movement of CD95 and its associated proteins into the lysosomal compartment. Analysis of DISC components revealed that recruitment of FADD, caspase-8 and -10 as well as caspase-8 activation peaked at 30 min, a time point at which most of the receptor had moved into an EEA-1-containing compartment. In contrast to type I ACHN cells, Rab4, EEA-1 and CatD demonstrated minimal increases following stimulation in type II HCT15 cells (Figure 4D). Moreover, FADD, caspase-8 and -10 were only weakly detected in CD95-containing membranes at 60 min. Together, these data suggest that in type I cells, both of hematopoietic and nonhematopoietic origin, most of the DISC components were recruited to CD95 after its internalization into endosomal/lysosomal compartments.

Colocalization of CD95, FADD and active caspase-8 on endosomes following CD95 stimulation

To complement the biochemical studies, we analyzed the subcellular localization of FADD in untreated and CD95L-activated BJAB cells by deconvolution microscopy. For FADD, both nuclear as well as cytoplasmic staining have been described (Perez and White, 1998; Gomez-Angelats and Cidlowski, 2003; Screaton *et al*, 2003; O'Reilly *et al*, 2004). In untreated BJAB and PBTs, FADD was preferentially detected within the nucleus based on its colocalization with DAPI nuclear staining (Supplementary Figure 6A, panels 1 and 4 and data not shown). While there was minimal overlap of staining for EEA-1 with FADD in untreated cells (panel 3), overlap of FADD and EEA-1 was readily detected 5 min following CD95L activation (panel 7). Subcellular fractionation studies confirmed a predominant nuclear localization of FADD in untreated BJAB cells and the nuclear and cytoplasmic distribution in CD95L-treated BJAB cells (Supplementary Figure 6B). In contrast, confocal and fractionation studies demonstrated no change in the nuclear and cytoplasmic distribution of FADD in untreated and CD95-stimulated Jurkat T cells (Supplementary Figures 6C and D).

Analysis of CD95 and FADD localization in untreated and CD95-stimulated BJAB cells further supported their colocalization on early endosomes following CD95L engagement. In untreated BJAB cells, CD95 was expressed at the PM and not co-localized with FADD (Figure 5A, panels 1–3). Within 2 min following CD95L stimulation, CD95 had formed microaggregates at the PM and FADD was readily detected in the cytoplasm (panels 6–8). At 15 min following CD95L engagement, when FADD association with CD95 was maximal in these cells (see Figure 1B), significant colocalization of CD95 and FADD was detected in a TfR^+ early endosomal compartment (Figure 5A, panels 11–15). Similarly, colocalization of CD95 and activated caspase-8 was readily detected within an $EEA\text{-}1^+$ compartment within 15 min following CD95L engagement (Figure 5B, panels 4–6, and Figure 5C) consistent with the analysis of CD95-containing receptosomes (see Figure 4).

The recruitment of DISC components to activated CD95 after internalization appears to be in contradiction to our previous report on a very rapid recruitment of FADD and caspase-8 to activated CD95 (Kischkel *et al*, 1995). However, these studies involved detergent lysis of cellular membranes causing destruction of intracellular compartments and did not permit discrimination of recruitment of DISC components to different cellular membranes. Hence, we utilized a biochemical approach that preserved intracellular membrane compartments by analyzing the subcellular localization of CD95 and its associated proteins after separating PM and endosomal membrane fractions. Using BJAB cells, CD95L activation resulted in a time-dependent decrease in the amount of CD95 within the PM fraction that was associated

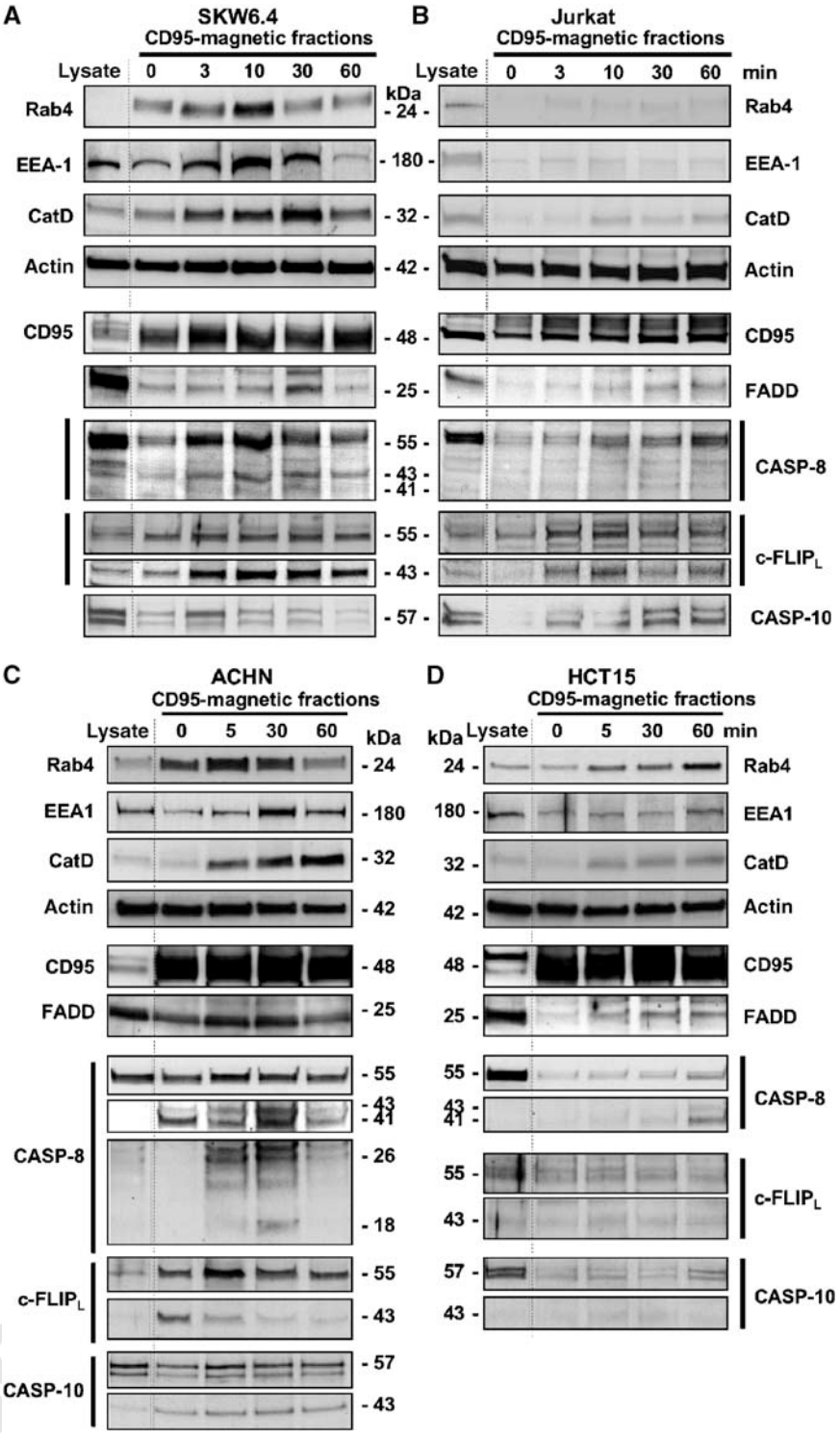

Figure 4 Internalization and endosomal maturation of CD95 DISC complexes. Time course of intracellular CD95-receptosome trafficking in SKW6.4 (**A**) and Jurkat (**B**) cells. Total cell lysates or magnetic fractions derived after 0, 3, 10, 30 and 60 min of anti-APO-1 mAb treatment were analyzed for signature proteins of endosomal maturation (Rab4 and EEA-1), lysosomes (CatD), actin, CD95 and DISC proteins. Note: twice as much lysate proteins were loaded to visualize Rab4, EEA-1 and CatD in the Jurkat cells. Time course of intracellular CD95-receptosome trafficking in ACHN (**C**) and HCT15 (**D**) cells. Total cell lysates or magnetic fractions derived after anti-APO-1 mAb treatment were analyzed as described in A and B.

with a concomitant increase of CD95 detected within the endosomal fraction (Figure 5D). While a low level of FADD was co-immunoprecipitated with ligand-bound CD95 in untreated BJAB cells, no significant increase in FADD or activated caspase-8 was co-immunoprecipitated with PM-associated CD95 following CD95L engagement. In contrast, increased amounts of FADD and caspase-8 were co-immunoprecipitated with activated CD95 within the endosomal membrane fraction (lanes 5–7), with kinetics consistent with observed internalization of CD95 in these cells.

Mutation of a putative AP-2-binding motif disrupts CD95 internalization and apoptosis

The cytoplasmic domain of CD95 contains a putative protein-sorting motif (Y^{291}DTL), consistent with the consensus YXXΦ AP-2-binding sequence (Ohno *et al*, 1995). Similar to the reduction in CD95 internalization in the AP-2 knockdown studies (Figure 3), internalization of a mutant CD95, in which Tyr 291 was changed to phenylalanine, following anti-CD95 mAb (CH-11) treatment, was diminished as compared to wt CD95 (Figure 6A). Concomitantly, the ability of CD95(Y291F) expressing cells to undergo apoptosis following treatment with an anti-CD95 mAb (CH-11) was similarly reduced (Figure 6B, lower two panels, and Figure 6C). As this tyrosine residue was localized within the FADD DD, we analyzed the *in vitro* ability of the DD of FADD to interact with the intracellular domains (ICDs) of wt CD95 or CD95(Y291F). ICDs derived from either wt or Y291F were similarly capable of binding *in vitro* translated FADD (Figure 6D). In contrast, the DD incorporating the mutation found in lpr^{cg} mice did not bind FADD (Martin *et al*, 1999). Hence, mutation of Tyr 291

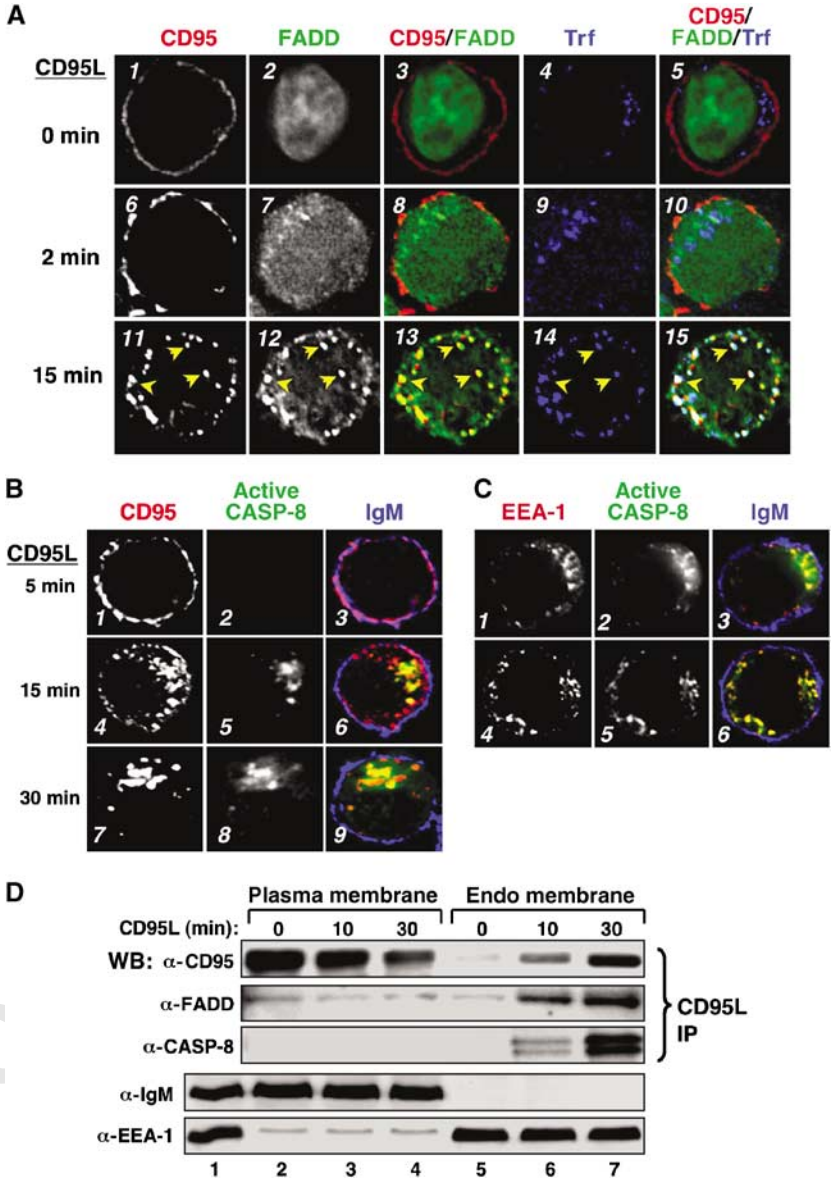

Figure 5 Colocalization of CD95, FADD and activated caspase-8 on early endosomes following CD95L stimulation. (**A**) BJAB cells were preincubated with Cy5-labeled transferrin (blue) for 15 min, left unstimulated or stimulated with Flag-CD95L for 2 or 15 min, then fixed and stained for CD95 (red) or FADD (green). Individual and overlay fluorescence are shown in deconvolution analysis. Data shown are representative of >200 cells analyzed. (**B**) Activated caspase-8 colocalizes with internalized CD95. BJAB cells were stimulated with Flag-CD95L. Cells were fixed and stained for CD95 (left), cleaved (active) caspase-8 (middle) or IgM. Merged images stained for CD95 (red), active caspase-8 (green) and IgM (blue) as a PM marker are shown on the right (panels 3, 6 and 9). Data shown are representative of >100 cells analyzed. (**C**) Activated caspase-8 co-localizes with EEA-1^+ endosomes. BJAB cells, treated with Flag-CD95L for 15 min, were fixed and stained for EEA-1 (left), activated caspase-8 (middle) or IgM. Merged images stained for EEA-1 (red), activated caspase-8 (green) and IgM (blue) are shown on the right (3 and 6). (**D**) BJAB cells were stimulated with Flag-tagged CD95L. PM (lanes 2–4) and endo membrane (lanes 5–7) fractions were separated from total cellular membrane extract (lane 1). Following fractionation, association of FADD and caspase-8 with activated CD95–CD95L complexes was analyzed by immunoblotting for FADD and caspase-8. Plasma and endo-membrane fractions were also immunoblotted for IgM as PM and EEA-1 as endosomal markers.

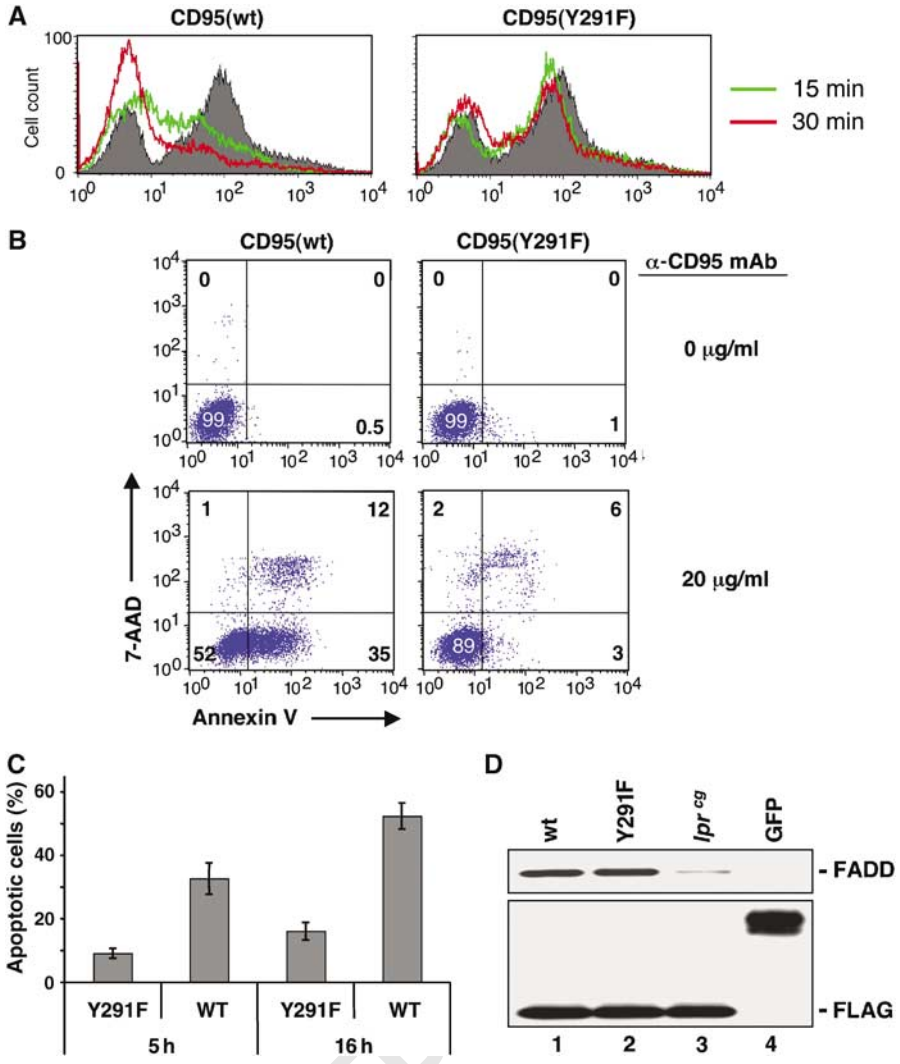

Figure 6 Internalization mutant of CD95(Y291F) is compromised in CD95L-mediated apoptosis. (**A**) A20 cells were transfected with wt human (h)CD95 or hCD95(Y291F). Cells were analyzed for CD95 downregulation following anti-hCD95 (CH11) activation at 0 (gray shadowed area), 15 (green) and 30 (red) min. (**B**) A20 cells were transfected with wt hCD95 or hCD95(Y291F). Cells were analyzed for apoptosis using the AnnexinV/7-AAD assay following anti-hCD95 (CH11) activation for 16 h. Data shown are representative of three experiments. (**C**) A20 cells were transfected with wt hCD95 or hCD95(Y291F). % apoptotic cells were quantified following incubation with an anti-hCD95 (CH11) mAb for 5 and 16 h. (**D**) CD95(Y291F) can bind FADD *in vitro*. The *in vitro* translated ICDs of wt CD95 (lane 1), internalization mutant CD95(Y291F) (lane 2), or the corresponding hCD95 mutation(V254N) in *lpr*^{cg} mice (lane 3) fused to a FLAG epitope was incubated with a biotinylated FADD DD. Flag-CD95-bound FADD was immunoprecipitated using anti-Flag mAb-coupled beads and analyzed by blotting for FADD and Flag. Flag-epitope-tagged GFP was used as a control protein (lane 4).

within the consensus AP-2-binding motif of CD95 compromised CD95L-mediated internalization and apoptosis, but not its ability to potentially interact with FADD. Together, our studies utilizing biochemical, genetic and imaging approaches indicate that CD95 internalization is required for efficient DISC assembly and activation of proapoptotic pathways.

CD95-mediated signaling independent of CD95 internalization

CD95 engagement in CD95-resistant tumor cells or in CD95 apoptosis-sensitive type I tumor cells treated with sCD95L has been demonstrated to activate nonapoptotic signaling pathways, including Erk and NF-κB (Ahn *et al*, 2001; Qin *et al*, 2002; Barnhart *et al*, 2004). While our data indicated that receptor internalization in type I cells was required for activation of proapoptotic pathways, we examined the requirement of receptor internalization for CD95L-induced nonapoptotic signaling pathways: Erk1/2 phosphorylation and NF-κB transcription activation. Cells transfected with control siRNAs and treated with Flag-CD95L demonstrated no significant Erk1/2 activation (Figure 7A, lanes 1–3). By contrast, cells transfected with AP-2($\alpha+\mu_2$) or CHC siRNAs showed CD95L-induced Erk1/2 activation (lanes 5–7 and 9–11). BJAB cells transfected with FynC-GFP-INP54p also induced a dose-dependent increase in NF-κB responsive luciferase reporter, while cells transfected with FynC-GFP control had no NF-κB response as all cells had undergone apoptosis following CD95L stimulation (Figure 7B and data not shown). In both experimental systems, Erk and NF-κB responses remained intact when cells were stimulated through the B-cell antigen receptor or with phorbol 12-myristate 13-acetate (PMA). Crosslinking of the internalization defective

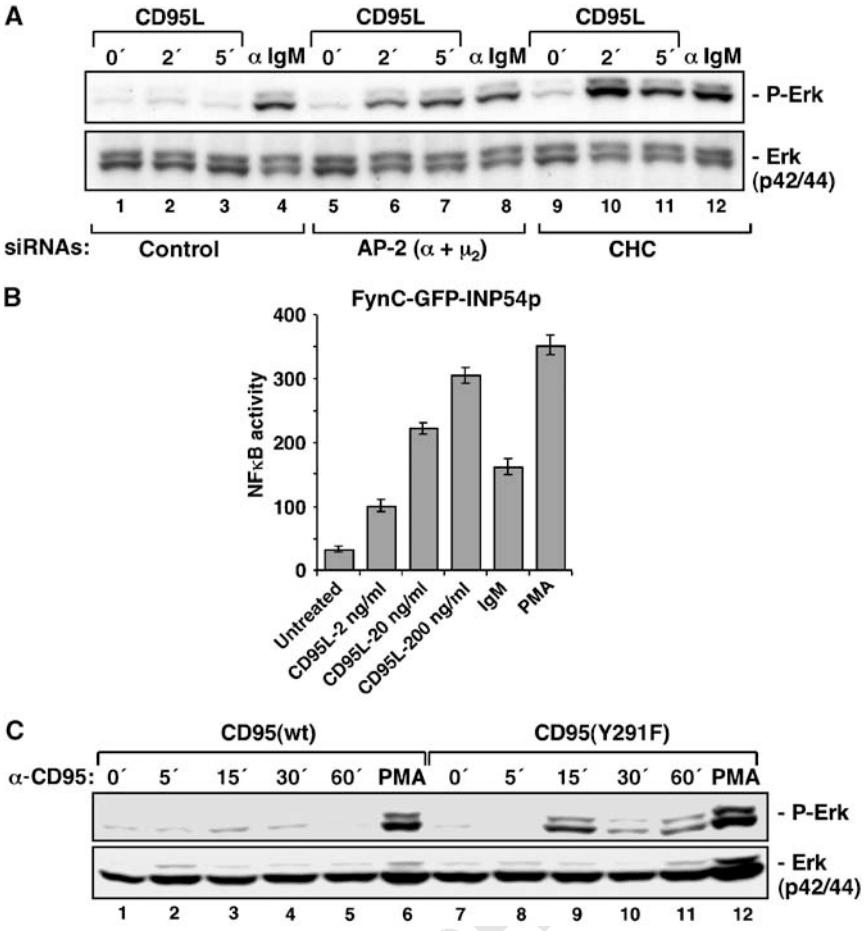

Figure 7 CD95L-mediated internalization-independent signaling. (**A**) Activation of Erk signaling pathway in cells transfected with AP-2($\alpha + \mu_2$) or CHC siRNAs. BJAB cells, transfected with control, AP-2($\alpha + \mu_2$) or CHC siRNAs, were incubated with Flag-CD95L for the specified times or anti-IgM Fab_2' for 5 min. Cell lysates were analyzed for phospho-Erk1/2 (top) or total Erk1/2 (bottom) expression. Data shown are representative of two experiments. (**B**) Activation of NF-κB transcription in FynC-GFP-INP54p$^+$ cells. BJAB cells transfected with FynC-GFP-INP54p were analyzed for transcriptional activation of NF-κB following Flag-CD95L, PMA or BCR stimulation. These data are representative of two experiments. (**C**) Activation of Erk signaling pathways by CD95(Y291F). A20 cells, transfected with wt CD95 or CD95(Y291F), were incubated with anti-hCD95 mAb (CH11) for the specified times. Cell lysates were analyzed for phosphor-Erk1/2 (top) or total Erk1/2 (bottom) expression. Cells were also treated with PMA as a positive control.

CD95(Y291F) mutant also induced Erk activation, even more efficiently than the wt receptor (Figure 7C).

Isotype switch variants of the anti-APO-1 mAb have been reported to exhibit markedly distinct cellular activities (Dhein *et al*, 1992; Oehm *et al*, 1992). While the widely used IgG3 anti-APO-1 mAb induced apoptosis of type I SKW6.4 and H9 cells, the IgG2b switch variant of anti-APO-1, with identical specificity and affinity for CD95, does not induce apoptosis (Dhein *et al*, 1992; Supplementary Figure 7A). Consistent with the ability of the IgG3 backbone to self-aggregate through Fc–Fc interactions to induce cytotoxicity, additional crosslinking of the IgG2b anti-APO-1 mAb with protein A resulted in cellular apoptosis (Supplementary Figure 7A). While the IgG3 anti-APO-1 mAb rapidly induced capping, clustering and internalization of CD95, the nonapoptotic IgG2b anti-APO-1 mAb was unable to induce capping or internalization of CD95 (Figures 8A and B), though the IgG2b anti-APO-1 was able to stain CD95 as small surface structures consistent with the described signaling protein oligomerization structures (SPOTS) (Muppidi and Siegel, 2004; Siegel *et al*, 2004). Moreover, the IgG3, but not the IgG2b, anti-APO-1 mAb induced DISC formation in type I SKW6.4 and H9 cells (Figure 8C, lanes 1–8). Hence, the ability of the anti-APO-1 mAb to induce DISC assembly and apoptosis was associated with the ability of the anti-APO-1 mAb to induce CD95 internalization.

We further analyzed whether CD95 could activate nonapoptotic pathways when stimulated by the IgG2b and IgG3 anti-APO-1 mAbs. MCF7(FV) and MCF7(FB) cells, which have been extensively characterized with respect to induction of nonapoptotic pathways through CD95 and the resulting functional consequences, were chosen for this analysis (Stegh *et al*, 2002). Isolation of magnetic internalizing vesicles demonstrated that MCF7(FV) cells activated by the IgG3 anti-APO-1 mAb maximally recruited DISC components, coincident with the presence of endosomal EEA-1 and Rab4 markers (Supplementary Figure 7B). Similarly, maximal processing of caspase-8 was detected 60 min after CD95 stimulation, again consistent with the requirement of receptor endocytosis for activation of proapoptotic signaling pathways. Consistent with the differential abilities of the two isotype anti-APO-1 mAbs to induce apoptosis in MCF7(FV) cells, the proapoptotic IgG3 anti-APO-1 mAb efficiently induced DISC assembly, while DISC assembly was not detected

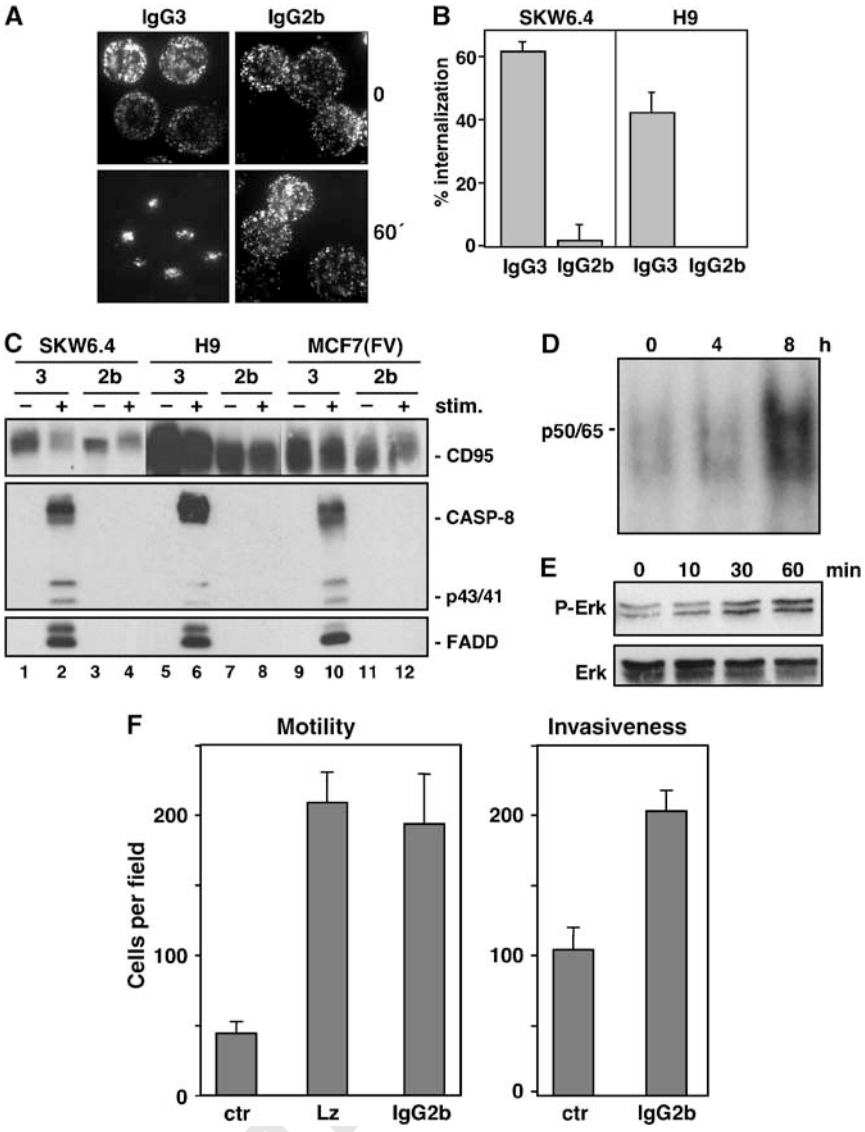

Figure 8 Activation of nonapoptotic signaling by anti-APO-1 IgG2b that does not induce CD95 internalization or apoptosis. (**A**) Effects of FITC-conjugated IgG3 (left two panels) and IgG2b (right two panels) anti-APO-1 mAbs on CD95 clustering in SKW6.4 cells was analyzed by fluorescence microscopy after 1 h of stimulation. (**B**) SKW6.4 cells were treated with either IgG3 or IgG2b anti-APO-1 mAbs for 1 h at 37°C. The number of cells with internalized CD95 following 1 h of Ab treatment was quantified. The experiment was performed in triplicates and is shown as the $\mu \pm$s.d. (**C**) SKW6.4 and H9 cells were treated with IgG3 (lanes 1, 2, 5 and 6) or IgG2b (lanes 3, 4, 7 and 8) anti-APO-1 mAbs. Activated CD95 molecules were immunoprecipitated and analyzed by Western blotting for CD95 (C20), caspase-8 and FADD. (**D**) EMSA analysis of MCF7(FV) cells using a ^{32}P-labeled oligonucleotide carrying NF-κB-binding sites stimulated with 1 μg/ml IgG2b anti-APO-1 for the indicated times. p50/p65 denotes the migration of the NF-κB heterodimer. (**E**) MCF7(FV) cells stimulated with 1 μg/ml IgG2b anti-APO-1 for the indicated times were analyzed for pErk and total Erk by Western blotting. (**F**) MCF7(FB) cells, treated with control (ctr), LzCD95L or IgG2b anti-APO-1 mAb, were analyzed using *in vitro* motility (left) or invasiveness (right) assays.

with the nonapoptotic IgG2b anti-APO-1 mAb (Figure 8C, lanes 9–12). However, treatment of MCF7(FV) with the noninternalizing IgG2b anti-APO-1 mAb induced both NF-κB and Erk activation (Figures 8D and E). Similar data were observed for ACHN and MCF7(FB) cells treated with the IgG2b anti-APO-1 mAb (data not shown). Finally, we tested the ability of both CD95L and non-apoptotic anti-APO-1 to modulate cellular motility and invasiveness. Both CD95 stimuli enhanced *in vitro* cellular motility and invasiveness using MCF7(FB) cells (Figure 6F). Hence, signaling through noninternalization-inducing stimuli can activate a multitude of signaling pathways that can contribute to enhanced tumorigenic potential, whereas internalization of CD95 is only required for apoptosis signaling.

Discussion

Receptor internalization is required for CD95L-induced apoptosis in type I cells

Formation of the DISC represents a critical step in the initiation of apoptosis induction through CD95 (Cremesti *et al*, 2001; Grassme *et al*, 2001; Algeciras-Schimnich *et al*, 2002). In this report, we utilized six distinct experimental approaches to demonstrate an unexpected role for receptor endocytosis in DISC assembly and apoptosis in CD95 signal transduction in type I cells. The first strategy involved selective modulation of PIP_2 levels that impaired internalization of CD95. The second strategy utilized siRNAs that target the clathrin endocytic machinery. The third involved the

concordance of different switch isotypes of an anti-APO-1 mAb to induce CD95 internalization, DISC formation and apoptosis. The fourth involved a selective internalization mutant in the cytoplasmic domain of CD95. The fifth utilized a novel technique to directly follow internalizing receptosomes containing CD95 and the sixth involved subcellular fractionation studies—all of which demonstrate that DISC assembly occurs predominantly after CD95 has internalized and has entered an early endosomal compartment. Additionally, confocal microscopy of activated cells indicates that CD95 co-localizes with FADD and activated caspase-8 on an EEA-1$^+$ compartment. While the initiation of DISC assembly may occur prior to and/or independent of receptor internalization, these data support an important role of CD95 internalization for efficient assembly of DISC components, activation of caspase-8 and -3, and induction of cellular apoptosis in type I, but not type II, cells. Hence, CD95 internalization in type I cells plays a requisite positive function rather than an inactivating function that would otherwise be biologically counterproductive for a cell destined for apoptosis.

A recent study has demonstrated that the assembly of activated TNF-R1 complexes with FADD, TRADD and caspase-8 was also dependent on receptor endocytosis (Schneider-Brachert *et al*, 2004). An internalization-deficient TNF receptor, while capable of recruiting RIP1 and TRAF2 at the PM, was unable to initiate DISC formation and induce apoptosis (Schneider-Brachert *et al*, 2004). Our study now provides the first evidence for a member of the subgroup of death receptors that directly recruit FADD (which includes CD95, DR4 and DR5), that the spatial and temporal regulation of membrane dynamics and internalization of receptors serves a critical regulatory function in defining cellular fate.

Receptor internalization and membrane-bound CD95L

Our studies also demonstrate a requirement for CD95 internalization in mCD95L-induced apoptosis. The ability of wt mCD95 and the mCD95L(DA4) mutant as well as covalently bound anti-CD95 mAb to induce internalization of CD95 and to activate apoptosis in BJAB and SKW6.4 cells supports the idea that CD95 must undergo some biophysical alteration induced by binding of ligand or agonistic mAbs. Receptor ligation results in the rapid formation of higher-ordered aggregates of CD95 within seconds following receptor cross-linking (Kischkel *et al*, 1995). High resolution confocal microscopy and live-cell imaging have recently revealed the formation of higher-ordered CD95 oligomers, termed SPOTS, at the PM following receptor engagement, a process that is dependent upon FADD association, but independent of caspase activation (Siegel *et al*, 2004). In addition, biochemical studies have demonstrated that CD95 is further recruited to raft membrane fractions, a process that is independent of its DD and DISC formation, following receptor activation (Eramo *et al*, 2004) (Figure 9). Hence, CD95L engagement induces spatial and conformational alterations in CD95 that permit receptor oligomerization, SPOT formation and localization within lipid rafts. Our studies define an additional requirement for the oligomerized CD95 to induce apoptosis: inter-

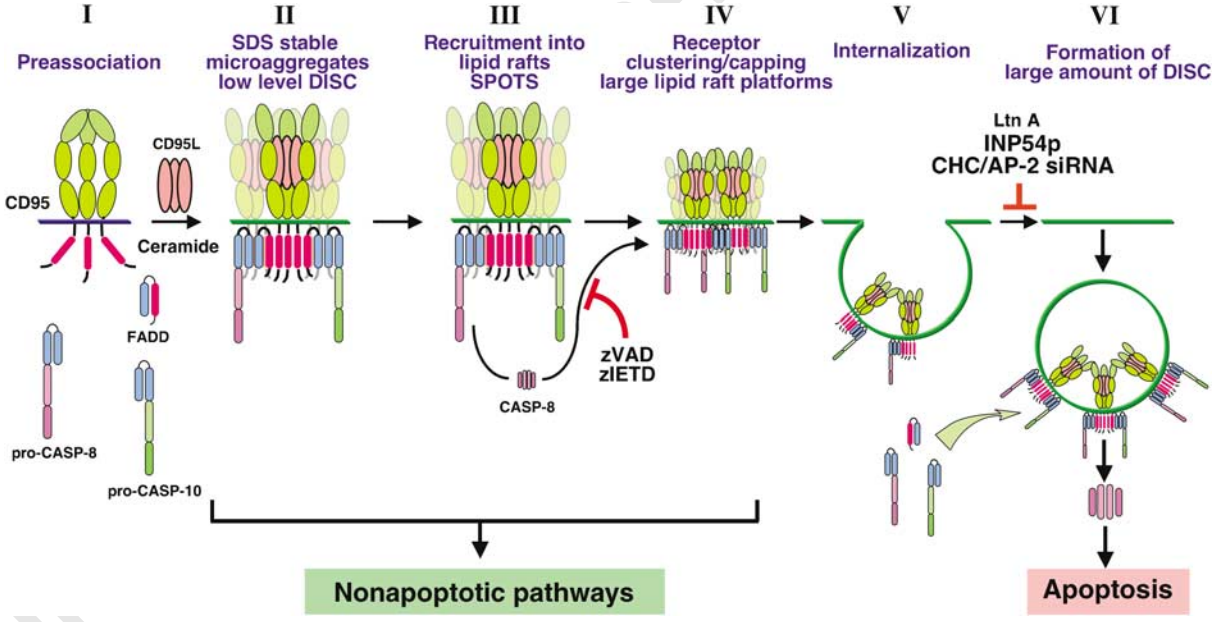

Figure 9 Model for early CD95 signaling. I, Ligand-independent receptor preassociation. II, Formation of microaggregates that are detected as SDS stable aggregates and low level of DISC formation. It was shown that cells expressing sphingomyelin synthase 1 form SDS stable aggregates more efficiently than SMS-negative cells (Miyaji *et al*, 2005), suggesting that ceramide, although not essential for CD95 signaling, is a general enhancer of CD95-mediated apoptosis. III, Recruitment of CD95 into lipid rafts to form SPOTS. IV, Receptor clustering also referred to as capping and formation of large lipid raft platforms. This step depends on generation of active caspase-8 by the DISC and can be efficiently prevented by preincubating cells with either zVAD-fmk or zIETD-fmk. In the absence of internalization, signals from steps 2–4 have the potential to activate nonapoptotic pathways, but are insufficient to kill type I cells. V, Internalization of the activated receptor. VI, Migration of internalized CD95 into an endosomal compartment and further recruitment of DISC components. This step is dependent on actin filaments since it can be inhibited by latrunculin A (LtnA). This step also requires PIP_2 and it is clathrin mediated, since it is inhibited by either overexpression of INP54p or downregulation of CHC or AP-2. In case of mCD95L-induced apoptosis, we postulate a ligand-induced internalization of an activated CD95 complex that no longer contains the ligand. DISC components are FADD/Mort1, caspase-8, caspase-10 and c-FLIP (not shown). Blue domains, DED; red domain, DD; the N-terminal PLAD in CD95 is shown in a different green tone.

nalization through a clathrin-mediated pathway and delivery to the early endosomal compartment for efficient DISC assembly and amplification (Figure 9).

Potential role of the endosome in CD95 association with FADD

Internalized CD95 within the endosome also appears to provide a localizing signal to concentrate FADD. In the absence of CD95 internalization, FADD remained in a diffuse cytoplasmic pattern and demonstrated substantially decreased localization within the EEA-1$^+$ early endosomal compartment (data not shown). Hence, DISC assembly or amplification on endosomes may provide additional temporal and spatial regulation to transport the activated DISC to downstream effectors. The requirement for activation of caspase-8 inside cells is consistent with a previous study that found that active caspase-8 generated at and tethered to the PM does not kill cells (Martin *et al*, 1998).

Recent evidence for a variety of receptors supports a role for the endosome in the control of signal transduction. In the case of the EGF receptor, Grb2 and activated Ras colocalize not only at the PM, but also on endosomes following EGF stimulation (Di Guglielmo *et al*, 1994). In addition, p14 and MP1 members of the protein superfamily of small subcellular adaptor proteins (ProfIAP) are preferentially localized within the late endosome and complex with MEK1/2 to facilitate Erk1/2 activation (Wunderlich *et al*, 2001; Teis *et al*, 2002). Finally, the APPL (Adaptor protein containing PH domain, PTB domain and Leucine zipper motif) Rab5 effector proteins, localized within a subpopulation of endosomes, link EGF and oxidative stress signals with chromatin remodeling and gene transcriptional regulation (Miaczynska *et al*, 2004a). In addition to EGFR, targeting of the TNF-R1-associated DISC complex to *trans*-Golgi vesicles has been recently demonstrated to activate the endolysosomal acidic, but not the PM localized neutral, sphingomyelinase (Schneider-Brachert *et al*, 2004). Hence, the endosome localized signaling complex appears to provide a subcellular nidus for signal amplification and routing to appropriate downstream effectors.

Compartmentalization of CD95 signaling is important in defining cellular outcome

Our studies also indicate that alterations in the ability of CD95 to internalize have profound effects in the cellular fate of CD95-activated cells. Cells unable to internalize CD95, through expression of FynC-GFP-INP54p, downmodulation of the clathrin-AP-2/CHC endocytic machinery or through the use of nonapoptotic anti-CD95 mAbs, induce transcriptional activation of NF-κB and activation of Erk1/2 following CD95 engagement. Hence, additional types of signaling likely occur independent of receptor internalization. Activation of nonapoptotic signaling pathways, including MAPK and NF-κB signaling pathways, by CD95L has been suggested to play a role in the tumorigenesis of CD95-resistant tumors (Ahn *et al*, 2001; Barnhart *et al*, 2004; Peter *et al*, 2005). Indeed, treatment of CD95L-resistant MCF7(FB) cells with anti-APO-1 mAb or soluble CD95L increased tumor cell motility and invasiveness. Hence, the dynamics of CD95 membrane localization and internalization plays a critical role to balance internalization-dependent apoptotic and internalization-independent nonapoptotic pathways to drive cellular apoptosis and other functions, respectively.

The membrane dynamics of TNF-R1 have also been demonstrated to play a critical role in defining the cellular fate of TNFR activation. TNF-R1-induced apoptosis involves sequential signaling complexes. The first involves the assembly of a lipid raft-localized complex involving TNF-R1, TRADD, RIP1 and TRAF2 (complex 1) and mediates NF-κB activation prior to ubiquitinylation and degradation of the TNF-R1 complex (Legler *et al*, 2003; Micheau and Tschopp, 2003). Complex 1 undergoes yet-to-be defined modifications such that the DD of TRADD and RIP1, previously bound to TNF-R1, become available to interact with other DD-containing proteins. The recruitment of FADD and caspase-8 to this modified cytoplasmic complex initiates the cellular apoptotic machinery (complex 2) (Micheau and Tschopp, 2003). Complex 2 forms inside cells and does not contain the receptor. Recent studies tracking receptor-containing internalized vesicles, however, demonstrate that the DISC remains bound to the internalized TNF-R1 to form endosomal 'death-signaling vesicles' (Schneider-Brachert *et al*, 2004). For CD95, stimulation by CD95L induces formation of low level of DISC at the cell surface followed by internalization of the entire complex (Figure 9). In type I cells, this internalization step triggers recruitment of large amounts of the signaling proteins FADD and caspase-8 to the activated receptor on endosomes executing apoptosis.

Together, our results provide support for the view that the subcellular location of receptor signaling plays important roles in defining cellular fates. In the case of CD95, receptor internalization commits the cell to a proapoptotic outcome by delivering the activated receptor through an endosomal signaling pathway. Conversely, inhibition of receptor internalization enables the activated receptor to activate biochemical pathways that induce prosurvival pathways. These dynamics are not only critical in understanding CD95 biology but also may have important implications in understanding the effects of chemotherapeutic agents that affect receptor trafficking in combination with emerging proapoptotic treatments in cancer therapy.

Materials and methods

Antibodies used in this study are summarized in Supplementary Table I. Additional reagents are summarized in Supplementary Table II. Methods for cells, molecular construction of cDNAs, protein expression, immunofluorescence and receptor capping are described in Supplementary Figure 8. Methodologies for CD95 activation, DISC analysis, membrane fractionation, apoptotic and *in vitro* invasiveness assays, siRNA preparation and transfections are described in Supplementary Figure 9.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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